New Neonatal Porcine Diarrhoea Syndrome

A study on its aetiology, epidemiology and clinical manifestations

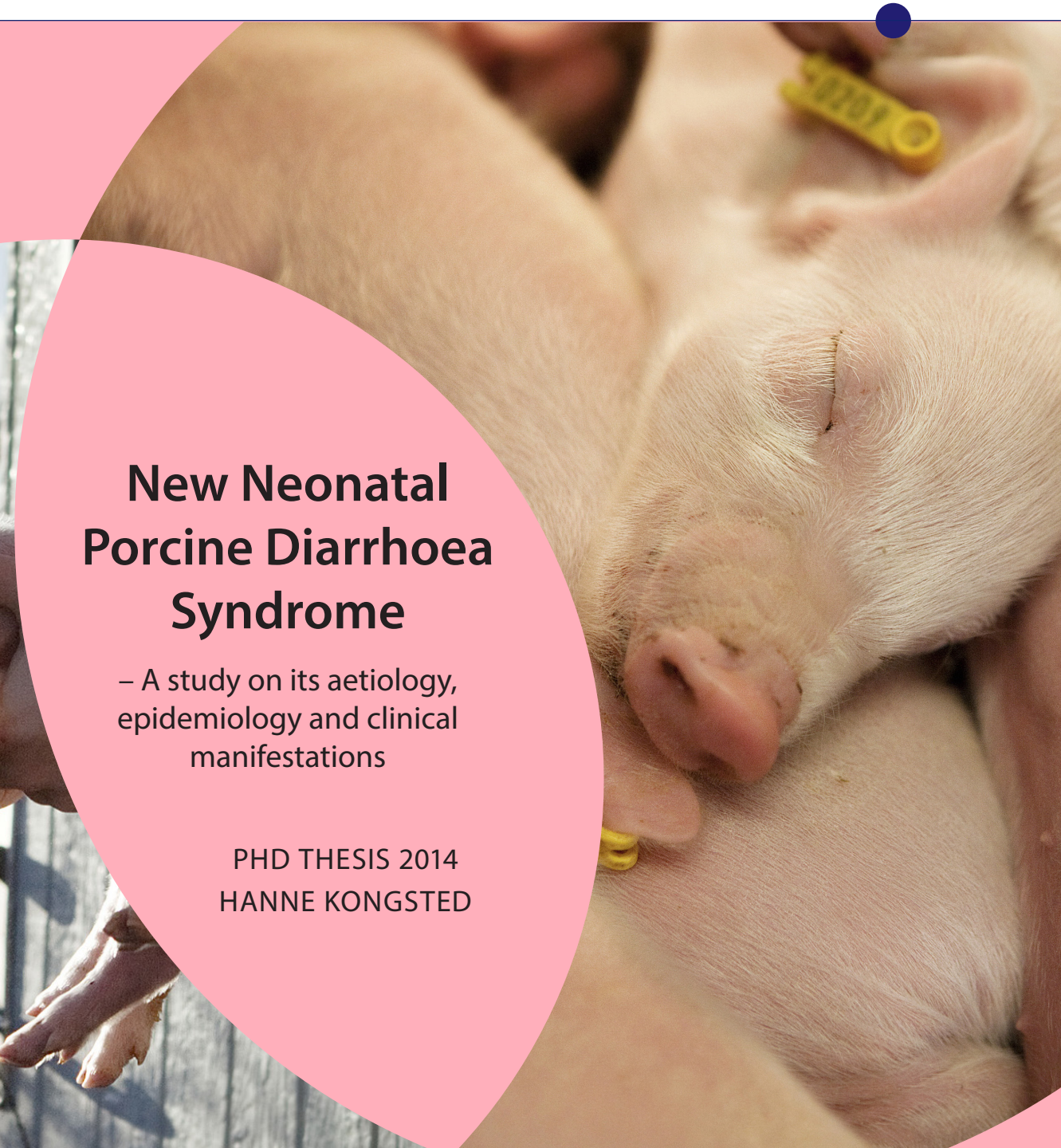
Kongsted, Hanne

Publication date:
2014

Document version
Early version, also known as pre-print

Citation for published version (APA):
Kongsted, H. (2014). *New Neonatal Porcine Diarrhoea Syndrome: A study on its aetiology, epidemiology and clinical manifestations*. (1 ed.) SL Grafik.

FACULTY OF HEALTH AND MEDICAL SCIENCES
UNIVERSITY OF COPENHAGEN



New Neonatal Porcine Diarrhoea Syndrome

– A study on its aetiology, epidemiology and clinical manifestations

PHD THESIS 2014
HANNE KONGSTED

New Neonatal Porcine Diarrhoea Syndrome

- A study on its aetiology, epidemiology and clinical manifestations

Hanne Kongsted

PhD thesis 2014

This thesis has been submitted to the Graduate School of the Faculty of Health and Medical Sciences, University of Copenhagen

Main supervisor: Jens Peter Nielsen
Department of Large Animal Sciences
Faculty of Health and Medical Sciences
University of Copenhagen

Co-supervisors: Birgitta Svensmark
Danish Pig Research Centre

Poul Bækbo
Danish Pig Research Centre

Sven Erik Jorsal
National Veterinary Institute
Technical University of Denmark

Henrik Elvang Jensen
Department of Veterinary Disease Biology
Faculty of Health and Medical Sciences
University of Copenhagen

Helle Stege
Department of Large Animal Sciences
Faculty of Health and Medical Sciences
University of Copenhagen

Assessment committee: Carsten Enevoldsen
Department of Large Animal Sciences
Faculty of Health and Medical Sciences
University of Copenhagen

John Haugegaard
MSD Animal Health

Magdalena Jacobson
Swedish University of Agricultural Sciences

Front cover: Sleeping piglets from Herd 1

Back cover: Piglets on their way to the laboratory

Photo: Martin Dam Kristensen

New Neonatal Porcine Diarrhoea Syndrome
– A study on its aetiology, epidemiology and clinical manifestations

ISBN 978-87-7611-772-6

Printed by SL grafik, Frederiksberg C, Denmark (www.slgrafik.dk)

Preface

This PhD-thesis is the result of work done in relation to a large research programme scrutinizing the background of New Neonatal Porcine Diarrhoea Syndrome (NNPDS). My role in the programme was to integrate information from the clinical situations in the herds with diagnostic information from piglets in order to obtain an overall view on the problem.

Working with an emerging disease has been truly challenging. Only recently I came to terms with the fact that my task was not to find out whether or not a new disease had in fact emerged. Prior to that, I had spent a lot of time struggling with the intellectual challenge on how to figure out if diarrhoeal symptoms in fact represent something new or rather represent a higher prevalence of something already well-known. Thanks to my university supervisor, Jens Peter Nielsen, I stopped such philosophical thinking and accepted the fact that my task was to find out what NNPDS was, and not if it was in fact new…

I realise that the thesis is rather lengthy. I apologize for the many pages and hope that it does not scare off busy practitioners or others who may have an interest in the subject. My ambition was to describe the work that was carried out in the research programme as well as to give a theoretical introduction to neonatal diarrhoea with a special focus on infectious agents. I intended not only to address researchers but hope the thesis could also be useful for other people with interest in the field.

I would like to thank the Danish Pig Research Centre for giving me the opportunity to become an industrial PhD-student and to join the research programme on NNPDS. I am also very grateful to my university supervisor Jens Peter Nielsen for help and guidance and for (to a certain point…) bearing over with my philosophical reflections on whether or not to use the term “New.”

Herd-owners and their staff deserve warm thanks for participation and willingness to help during the field studies.

Thanks to my colleagues in Kjellerup - especially Birgitta Svensmark, Svend Haugegaard and Bo Sørensen - for continuing positivity and willingness to help – even when things turned out to happen during weekends. All laboratory technicians involved in the project deserve thanks for their enthusiasm and helpfulness in making things work out. I would also like to appreciate Svend and Birgitta for all our nice nerdy discussions on diarrhoea and pathology.

Big thanks to Nils Toft for guidance on epidemiological issues and for giving good input from outside the (sometimes weird) veterinary circles. Sigge Birkenfalk deserves gratitude for a truly memorable summerschool on communication. Finally, I would like to warmly thank my sweet sisters, Alice and Anette, for mental and moral support. Alice – thanks for all our meetings on Skype – for morning songs as well as for discussions on advanced statistics and support regarding layout…

Summary

The subject of this thesis is New Neonatal Porcine Diarrhoea Syndrome, which is a newly emerged syndrome not previously described. Investigations carried out in four herds with persisting undiagnosed problems on neonatal diarrhoea formed the basis of all the studies in the project.

The first part of the thesis gives a theoretical introduction to enteral disease in neonatal piglets with special focus on infectious agents related to or suspected to be related to diarrhoea in this age group.

The primary focus of the study was to evaluate in a case-control study if any infectious agents were associated with diarrhoea. A total of 101 piglets (51 diarrhoeic and 50 non-diarrhoeic) at the age of 3-7 days were euthanized for necropsy. All 51 diarrhoeic piglets had milk-filled stomachs, thus, starvation did not seem to cause the symptoms. The most prevalent gross lesion associated with diarrhoea was flaccidity of the intestines. Histologically, villous atrophy was the predominant lesion.

Samples from the euthanized piglets were submitted for aerob and anaerob bacteriological culture, detection of bacteria (specific and unspecific) by flourescence in situ hybridization (FISH), virulence gene determination of *E. coli* isolates, *Clostridium perfringens* (beta-2) toxin detection by enzyme-linked immunosorbent assay (ELISA) as well as detection of rotavirus A by ELISA and polymerase chain reaction (PCR) for coronavirus. The study did not point out any infectious agent explaining the syndrome across herds. The prevalence of *Clostridium perfringens* type A was higher in the non-diarrhoeic piglets than in the diarrhoiec piglets, and detection of beta-2 toxin was not statistically associated with diarrhoeic status. Within one herd, however, *E. coli* carrying EAST-1 genes as the only virulence factor and *Enterococcus spp.* were associated with diarrhoea.

In parallel with the case-control study, a cross sectional study with follow up was carried out. A total of 989 piglets from 86 litters in the four herds were weighed at birth and clinically examined daily until five days of age. In the same period, faecal consistencies were evaluated on a daily basis using rectal swabs. All piglets that died in the period between birth and day ten of life were necropsied. On day ten all piglets were weighed again. All sows included in the study (29 first parity and 57 second to seventh parity) were clinically examined on the day of parturition.

Many piglets in the study (approximately 30%) were diarrhoeic for a single day only. Approximately half of these were diarrhoeic on the day of birth. These findings encouraged to investigate whether diarrhoea on the day of birth might be a normal phenomenon un-related to the syndrome. A study on the effect of diarrhoea on average daily gain (ADG) concluded that diarrhoea restricted to the day of birth did not seem to have a negative effect on piglets. However, a risk-factor study concluded that faecal consistency on the day of birth was weakly associated with developing diarrhoea later on. Overall, it was concluded that diarrhoea on the day of birth should not automatically be interpreted as the first symptom of NNPDS, since in many cases this phenomenon seemed to be a normal physiological reaction. For practicalities, in the remaining part of the study, NNPDS was defined as diarrhoea at some point during the second to fifth day of life.

The ADG in piglets of the study was negatively influenced by a low birth weight and by NNPDS. NNPDS for a single day and for 2-5 days negatively affected ADG by 9 and 14 g, respectively. Being part of a severely affected litter negatively influenced individual piglets by 38 g per day. ADG was not influenced by herd of origin, parity of sow, gender or the presence of skin abrasions.

The study did not find any effect of NNPDS on mortality when herd of origin, gender and birth weight were taken into account. In one of the herds (Herd 1), however, 25% of the diarrhoeic piglets died, and it was concluded that the excess mortality in this herd (odds for dying in this herd was almost twelve times as high as in the herd with the lowest mortality) seemed to be due to diarrhoea. Male gender and low birth weight both increased odds for dying. Mortality was not significantly influenced by parity of sow or the presence of skin abrasions.

Concerning the development of NNPDS, parity was the only sow-level risk factor recognised. Neither litter size, number of stillborn nor clinical disease on the day of parturition were significant risk-factors. Low birth weight of the piglet and liquid faecal consistency on the day of birth were minor risk-factors for the development of NNPDS. Gender and clinical signs of failure to thrive on the day of birth (hollow flanks and rough hair coats) were not risk-factors.

The course of NNPDS varied between herds. In two of the herds, half of all cases started on day two of life, whereas in the other herds, symptoms often appeared a bit later. NNPDS was observed in a total of 60% (range: 39%-89%) of first parity piglets and 36% (range: 19-65%) of piglets born by mature sows. In most cases diarrhoea lasted for one to two days (of the five days of examination), but a few piglets (especially first parity piglets and piglets within Herd 1) were affected for up to four days. In all herds, first parity piglets were most severely affected, but apart from this, no overall tendency for diarrhoea to cluster within litters was observed. Causes of death varied between herds. In Herd 1, more than 70% of the necropsied piglets were diagnosed with enteritis, whereas in the remaining herds 15-30% of necropsied piglets had enteritis.

In terms of pathology and microbiology the study suggested that all four study-herds seemed to be affected by a new syndrome; NNPDS. The syndrome was preliminarily defined as: Non-haemorrhagic diarrhoea during the first week of life, with no association to known infectious agents and characterized by milk-filled stomachs and flaccid intestines at necropsy.

In terms of herd-epidemiology it was concluded that the four herds experienced outbreaks of neonatal diarrhoea with different clinical manifestations. The background for the marked differences in severity of symptoms was not explained by factors addressed in the study or by obvious problems in management. At the current stage of investigation the epidemiologic conclusion would be that NNPDS manifests very differently in different herds. Further research might, however, be able to establish that the four herds in fact were affected by problems of distinct aetiologies.

Sammendrag

Emnet for afhandlingen er Nyt Neonatalt Porcint Diarré Syndrom (NNPDS), som er et nyligt opstået syndrom, der ikke tidligere er beskrevet. Grundlaget for undersøgelserne i afhandlingen var fire besætninger, der gennem en længere periode havde haft problemer med neonatal diarré af ukendt årsag.

Første del af afhandlingen er en teoretisk introduktion til tarmsygdomme hos neonatale grise med særligt fokus på infektiøse agens med betydning for eller som er mistænkt for at have betydning for udvikling af diarré i denne aldersgruppe.

Det primære fokus i projektet var vha. et case-kontrol studie at vurdere om infektiøse agens kunne forklare symptomerne. I alt 101 grise på 3-7 dage (51 med og 50 uden diarré) blev aflivet til obduktion. Alle 51 grise med diarré havde mælke-fyldte maver ved obduktion, hvilket indikerede at symptomerne ikke var forårsaget af sult. Den mest udbredte makroskopiske læsion hos diarré-grise var atoni af tarme. Villus atrofi var den gennemgående histologiske læsion.

Materiale fra de aflivede grise undergik følgende undersøgelser: aerob og anaerob bakteriologisk dyrkning, virulensfaktor-bestemmelse af *E. coli* isolater ved polymerase Chain Reaction (PCR), flourescence in situ hybridisering (FISH) med specifikke og uspecifikke bakterie-prober, detektion af *Clostridium perfringens* beta-2 toksin ved Enzyme Linked Immunosorbent Assay (ELISA) samt ELISA for rotavirus A og PCR coronavirus. Undersøgelserne påviste ikke noget infektiøst agens som var associeret med diarré på tværs af besætninger. Forekomsten af *Clostridium perfringens* type A var højere i grise uden end i grise med diarré, og forekomsten af beta-2 toksin var ens i grise med og uden diarré. I én besætning var *E. coli* bakterier med EAST-1 som eneste virulensfaktor og *Enterococcus spp.* associeret med diarré.

For at få et indblik i besætnings-epidemiologien i forbindelse med syndromet, blev der parallelt med case-kontrol undersøgelsen gennemført en tværsnits-undersøgelse med opfølgning i de fire besætninger. I alt 989 grise i 86 kuld blev vejet ved fødslen og klinisk undersøgt dagligt ind til dag 5. I samme periode blev fæces-konsistensen evalueret dagligt ved hjælp af rektal-svabre. På dag 10 blev grisene vejet igen, og i perioden fra fødsel til dag 10 blev alle grise, der døde, obduceret. Alle søer (29 første paritet/ 57 anden til syvende paritet), der indgik i undersøgelsen, blev klinisk undersøgt på faringsdagen.

Mange grise (ca. 30%) i undersøgelsen havde kun diarré en enkelt dag. Omtrent halvdelen af disse havde diarré på den dag, de blev født. Disse resultater motiverede en undersøgelse af om diarré på dag ét evt. kunne være et normalt fænomen, som ikke skulle tillægges betydning i forbindelse med NNPDS. En undersøgelse af hvordan diarré påvirkede den gennemsnitlige daglige tilvækst konkluderede, at diarré begrænset til dag ét ikke havde en negativ indvirkning på grisene. Et risiko-faktor studie konkluderede imidlertid, at fæces-konsistensen på dag ét var svagt associeret med senere udvikling af diarré. Det blev sammenfattende konkluderet at diarré på dag ét lod til at være et normalt og uskadeligt fænomen hos nogle grise og ikke automatisk skulle fortolkes som det første symptom på NNPDS. Af praktiske hensyn blev NNPDS efterfølgende defineret som diarré i perioden mellem andet og femte levedøgn.

I undersøgelsen var den daglige tilvækst negativt påvirket af en lav fødselsvægt og af NNPDS. NNPDS en enkelt dag og i to til fem dage reducerede gennemsnitligt tilvæksten med hhv. 9 og 14 g pr dag. At være en del af et diarrékuld påvirkede grisene negativt med 38 g pr dag. Tilvæksten var ikke påvirket af besætning, soens paritet, grisens køn eller tilstedeværelsen af sår.

Der blev ikke fundet nogen sammenhæng mellem NNPDS og dødelighed når besætning, køn og fødselsvægt blev taget i betragtning. I én af besætningerne (Besætning 1) døde 25% af de grise der havde diarré, og det blev konkluderet, at overdødeligheden i denne besætning (odds for at dø i denne besætning var næsten tolv gange så høj som i besætningen med den laveste dødelighed) syntes at skyldes diarré. Hankøn og lav fødselsvægt var begge forbundet med forøget dødelighed. Dødeligheden var ikke påvirket af soens paritet eller tilstedeværelsen af sår.

Med hensyn til udvikling af NNPDS var paritet den eneste identificerede so-risikofaktor. Hverken kuldstørrelse, antal dødfødte eller klinisk sygdom på faringsdagen var forbundet med udviklingen af NNPDS. En lav fødselsvægt hos grisene og tynd gødningskonsistens på dag ét var begge svage risiko-faktorer for udvikling af NNPDS. Hverken køn eller kliniske tegn på mistrivsel på dag ét (indsunkne flanker og mat hårlag) var forbundet med udviklingen af NNPDS.

Forløbet af diarré varierede mellem besætninger. I to af besætningerne startede halvdelen af alle tilfælde på levedag 2, hvorimod symptomerne ofte begyndte lidt senere I de to andre besætninger. Ialt 60% af gyltegrisene (39%-89% inden for besætninger) og 36% af de ældre søers grise (19%-65% inden for besætninger) udviklede NNPDS. I de fleste tilfælde varede diarréen i én til to dage (ud af de fem undersøgelsesdage), men nogle få grise (især gyltegrise og grise i Besætning 1) havde symptomer i op til fire dage. I alle besætninger var gyltegrise mest påvirkede men ud over dette sås ingen generel tendens til at symptomerne udbredtes kuldvist. Dødsårsager varierede mellem besætningerne. I Besætning 1 fik over 70% af de selvdøde grise post-mortem diagnosen enteritis, hvorimod denne diagnose kun blev tildelt 15-30% af de selvdøde grise I de resterende besætninger.

I forhold til patologiske og mikrobiologiske fund viste studiet overordnet set, at de fire undersøgelsesbesætninger alle var påvirket af et nyt syndrom; NNPDS. Syndromet blev præliminært defineret som: Non-haemorrhargisk diarré i den første leveuge som ikke skyldes kendte infektiøse agens og som karakteriseres ved mælke-fyldte ventrikler og atoniske tarme ved obduktion.

Med hensyn til besætnings-epidemiologi manifesterede diarré-udbruddene i de fire besætninger sig meget forskelligt. Baggrunden for disse forskelle blev ikke forklaret af undersøgelser i studiet og umiddelbart heller ikke af driftsforhold. På det nuværende forsknings-stadie er den epidemiologiske konklusion at NNPDS ser ud til at manifestere sig meget forskelligt i forskellige besætninger. Yderligere undersøgelser vil måske senere fastslå, at problemerne i besætningerne faktisk havde forskellig aetiologi.

Photo: Martin Dam Kristensen

Contents

Photo: Martin Dam Kristensen

1 Introduction

1.1 Motivation

In 2010, I was engaged as an industrial Phd-student by the Danish Pig Research Centre, Danish Agriculture & food Council (DPRC). My task was to help solving an urgent problem on neonatal diarrhoea in Danish pig herds.

When the project was initiated, a couple of years had passed with frustrated veterinarians and farmers contacting national experts about severe outbreaks of neonatal diarrhoea, that did not respond to routine vaccinations or antibiotic treatment (personal communications, S.E. Jorsal, the National Veterinary Institute, Technical University of Denmark (DTU-Vet) and Birgitta Svensmark, DPRC). Antibiotic treatments which were reported to be unsuccessful included most commercially available types of antibiotics, and in many herd-cases several treatment regimes had been unsuccessfully tried out. In most laboratory submissions on neonatal diarrhoea, routine testing protocols did not explain the aetiology (Svensmark, 2009).

Thus, the starting point of research was that something new seemed to be going on, and we needed to figure out what. A collaborative research programme involving DTU-Vet, University of Copenhagen and DPRC was initiated. The research programme focused on infectious causes of diarrhoea, since experience from practice indicated that herd-immunity seemed to develop.

Programme activities included three Phd-studies with the following focuses:

1. To establish the clinical and gross pathological hallmarks of the disease and evaluate associations with microbial agents and to describe the herd- epidemiology of the disease (the present project)
2. To describe microscopic intestinal lesions associated with the disease and to evaluate associations with microbial agents detected by Fluorescence In Situ Hybridisation (FISH) (Phd-student: Beata Jonach, DTU-Vet)
3. To clarify the role of the bacterial microbiota in the development of the disease (Phd-student: Marie Louise Hermann-Bank, DTU-Vet)

The approach was to select a few herds expected to be representative of the disease, and to carry out a multitude of diagnostic examinations on piglets selected from these herds. Selection of herds and inclusion of piglets within the herds was carried out as a part of the present project.

The thesis focuses on pathology, microbiology and epidemiology in NNPDS, however, does not include results from studies on the general microbiota since the Phd-project focusing on that part is not yet concluded.

In parallel with the research in the programme, a survey on neonatal diarrhoea was carried out among swine practitioners (Kongsted, 2013). In this survey, participants were asked if they believed that a new disease entity had emerged during the later years. A total of 80% (63/79) fully or partly believed so. Of these, 60% (n=38) answered that the problems seemed to start around 2008.

My personal motivation for joining the research programme was the practical importance of the task and an intention to help farmers dealing with the problems. Being a former swine practitioner and current laboratory diagnostician, the broad task of combining clinical and epidemiological observations in herds with specific laboratory diagnostics in piglets appealed to me. First and foremost I was eager to find out if characteristic pathological and microbiological hallmarks of this new disease would appear.

1.2 Scientific aims of the thesis

Two aims were defined:

1. To define characteristics of New Neonatal Porcine Diarrhoea Syndrome (NNPDS) in individual piglets and thereby suggest a case-definition
2. To describe epidemiologic characteristics of NNPDS.

The objectives identified to fulfil these aims were;

Ad 1:

1. To describe gross findings and histological lesions in diarrhoeic vs. non-diarrhoeic piglets from selected problem-herds
2. To evaluate associations between the detection of the bacterial agents *Escherichia coli*, *Clostridium perfringens* type A/C , *Clostridium difficile* and *Enterococcus spp.* and diarrhoea in piglets across and within herds suspected to suffer from NNPDS
3. To evaluate associations between the detection of *Clostridium perfringens* beta2-toxin and diarrhoea in piglets across and within herds suspected to suffer from NNPDS
4. To evaluate associations between the demonstration of parasites in intestinal tissue and diarrhoea in piglets across and within herds suspected to suffer from NNPDS
5. To evaluate associations between the detection of rotavirus A and coronavirus and diarrhoea in piglets across and within herds suspected to suffer from NNPDS

Ad 2:

1. To describe the course of disease in four herds affected by NNPDS in terms of prevalence, timing and duration of diarrhoea
2. To specify time-periods of importance for the clinical diagnosis of NNPDS
3. To identify sow and piglet-level risk-factors associated with NNPDS
4. To evaluate the effect of NNPDS on weight gain and mortality

1.3 Outline of the thesis

Chapter 2 contains a theoretical introduction to neonatal enteral disease in piglets with a special focus on infectious agents.

Chapter 3 and 4 describe methods and results of the project, aiming at giving the full picture of the project and especially highlighting issues that are not dealt with within manuscripts. Methods and results that are

described within the manuscripts are only briefly summarized, whereas supplemental studies not published in any manuscripts are more thoroughly described.

Chapter 5 contains the three main manuscripts of the project;

1. Microbiological, pathological and histological findings in four Danish pig herds affected by a new neonatal diarrhoea syndrome
2. The effect of New Neonatal Porcine Diarrhoea Syndrome (NNPDS) on average daily gain and mortality in four Danish pig herds
3. Risk factors and epidemiological characteristics of new neonatal porcine diarrhoea syndrome in four Danish herds

and also includes a fourth manuscript;

4. No evidence of viral involvement in the new neonatal porcine diarrhoea syndrome in Danish pigs.

The fourth manuscript was included in order to present the whole battery of diagnostic examinations in the research programme. My contribution to this manuscript was limited and the diagnostic methods used in this manuscript are not dealt with in the text-part of the thesis.

Chapter 6 contains a general discussion of the study, putting pathological, microbiological and epidemiological evaluations together and into perspective.

Chapter 7 and 8 provide conclusions and perspectives of the study.

Photo: Martin Dam Kristensen

2 Introduction to neonatal enteral disease

Enteral disease in neonatal pigs (throughout the thesis I define the term "neonatal" as the first week after birth) may be the result of functional or inflammatory disturbances and is generally considered to be multifactorial. The primary focus of the thesis is infectious causes of diarrhoea, and therefore functional aspects are not discussed in depth. However, a few functional considerations of overall importance in relation to neonatal enteral infection are highlighted here;

- The placental transfer of immunoglobulins is negligible in pigs, and therefore newborn piglets depend on a sufficient transfer of colostral immunoglobulins in order to resist infection.
- Newborn piglets are sparsely covered with hair, lack adipose tissue and have a large body surface to body weight ratio. Low ambient temperatures direct energy resources away from the intestinal growth and maturation (Sangild, 2001) and decrease peristalsis thus delaying the passage of bacteria and protective antibodies through the intestine (Gyles and Fairbrother, 2004).
- The acid production in the stomach is lower than in adult animals and the milk-based diet further buffers the gastric fluid pH, altogether resulting in a decreased capacity to kill pathogens (Manners, 1976).
- Trypsin inhibitors present in colostrum, which prevent the degradation of colostral antibodies by trypsin, also prevent the degradation of toxins (eg. clostridial toxins).
- The regenerative capacity of neonatal intestinal epithelium is slower and the production of digestive enzymes in neonatal enterocytes is lower than in adult pigs. Insufficiency of digestive enzymes prevents the degradation of harmful substances, and the slow epithelial turn-over decreases the ability of the mucosa to regenerate (Sangild, 2001).
- At birth, intestines must adapt from placental to enteral nutrition. This process involves absorptive, digestive and immunological changes, posing a challenge to both functionality and the capacity to resist infection (Jacobi and Odle, 2012; Sangild, 2001). The complex processes involved in intestinal maturation have been intensively investigated in preterm piglets used as models for preterm babies (for a review, see Sangild et al., 2013).

Altogether, these factors contribute to the vulnerability of neonatal animals to enteral disease.

2.1 Establishment of the normal microbiota

In adult animals, the intestinal microbiota, which is a complex mix of bacteria, fungi, protozoa, yeasts and bacteriophages, serves as a barrier for colonisation and proliferation of invading pathogens. Furthermore, the resident microbiota influences nutrient absorption, lipid and carbohydrate metabolism and stimulates the regenerative capacity of tissue (Lewis et al., 2010).

In neonatal animals, the developing microbiota contributes to immune development and immune adaptation, and disturbances in the colonization process are known to play a role in the immune dysregulation

associated with necrotizing enterocolitis in preterm pigs (Sangild et al., 2013). Such disturbances, possibly induced by routine post-natal antibiotic treatments, may also play a role in herd cases of neonatal enteral disease.

2.2 Diagnosis of infectious enteral disease in neonatal piglets

The traditional approach towards diagnosing the cause of an infectious pig disease is

1. To evaluate the clinical picture in the herd with respect to timing, prevalence and severity in the light of the immunological profile and vaccination strategy of the herd
2. To evaluate the clinical appearance of individual pigs
3. To evaluate gross lesions and
4. To perform relevant laboratory diagnostics

Dealing with neonatal diarrhoea is complex because

Ad 1. Clinical pictures in herds suffering from neonatal diarrhoea of different aetiologies are not clearly distinguishable (Thomson and Friendship, 2012). Timing and spread of disease depend on individual herd factors that may not be obvious or recognized by the herd manager. Furthermore, the epidemiological pattern of disease is usually hard to assess due to the heavy use of cross-fostering in modern herds.

Ad 2. In individual pigs, neonatal diarrhoea is usually yellow and liquid independent of the underlying aetiology. The effect on body condition and hydration status as well as the fatality of a given disease depends a lot on the quality and availability of colostrum, local environment and the support offered by staff persons.

Ad 3. Lesions in cases of neonatal diarrhoea are often unspecific and independent of the aetiology (Brown, et al., 2007).

Ad 4. The detection of agents within the establishing microbiota is troublesome. The microbiota seems to establish differentially from herd to herd (Bailey et al., 2013) and it is hard to tell whether vigorous growth of a certain agent in a specific herd-case is associated with disease or simply a reflection of the normal colonisation process.

2.3 Infectious agents in neonatal diarrhoea

In the context of investigating an apparently new and probably multifactorial syndrome like NNPDS, both well-known agents and yet un-established agents are relevant to consider. In the following literature-review much focus will be given to un-established agents. The intention is to provide an overview of the available literature on infectious agents related to neonatal diarrhoea in piglets.

Infectious agents are presented in three separate sections;

1. Agents with a well-established clinical importance
2. Clostridia of uncertain clinical importance (*Clostridium perfringens* type A and *Clostridium difficile*)

3. Agents with unknown clinical importance

Clostridium perfringens type A and *Clostridium difficile* are given special attention since both are relatively well investigated, in relation to neonatal diarrhoea in pigs. This contrasts the agents in section 3, which have only been sparsely investigated in this context.

2.3.1 Agents with a well-established clinical importance

Enterotoxigenic Escherichia coli, *Clostridium perfringens* type C, transmissible gastroenteritis virus, porcine epidemic diarrhoea virus, rotavirus A and *Cystoisospora suis* (previously *Isospora suis*) are agents with known clinical importance in herd-outbreaks of neonatal diarrhoea.

Bacteria

Enterotoxigenic Escherichia coli

Escherichia coli (*E. coli*) are gram-negative, facultative anaerobic bacteria, which colonize the intestinal tract. Enterotoxigenic *E. coli* (ETEC) contain genes for fimbriae and enterotoxin and are the classical E. coli bacteria associated with diarrhoea in pigs (Fairbrother and Gyles, 2012).

Clinical manifestations and relevance

Neonatal E. coli diarrhoea is seen in piglets at the age of 0-4 days, and affects single piglets or whole litters. In individual piglets, clinical manifestations vary from mild without evidence of dehydration to profuse watery diarrhoea and vomiting. Dehydration may lead to death before the development of diarrhoea (Fairbrother and Gyles, 2012).

In modern swine production, vaccination of sows pre-farrowing with vaccines containing *E.coli* fimbrial antigens (F4, F5 and F6) is common. Furthermore, in 2003 a genetic selection programme on F4 resistance was initiated in the Danish breeding nucleus (Anonymous, 2010). Today, close to 100% of breeding boars are resistant to F4, and the effect in production herds should be evident. Overall, the clinical importance of neonatal *E. coli* diarrhoea seems to have decreased during the later years, as suggested by surveys conducted on material obtained from general herds and laboratory submissions (Chan et al., 2013; Cruz Jr et al., 2013; Svensmark, 2009).

Pathogenesis

ETEC exert their effect on small intestinal epithelial cells by means of enterotoxins (STa, STb and LT), which induce functional changes leading to hypersecretion (STa and LT) and malabsorption (STb) (Nagy and Fekete, 1999). Intimate contact with the intestinal mucosa is a prerequisite for colonisation and production of enterotoxins (Gyles and Fairbrother, 2004), and different fimbrial adhesion factors have been demonstrated. In neonatal piglets, fimbrial types F4, F5, F6, F17, F18 and F41 have been associated with diarrhoea (Ojeniyi et al., 1994).

Pathology

At necropsy intestines are flaccid and contents are fluid. Histologically, the functional changes induced by enterotoxins are not accompanied with morphological changes, but layers of bacteria can be detected adherent to the small intestinal mucosa (Brown et al., 2007).

Diagnosis

A diagnosis of enteric colibacillosis is normally assigned based on bacterial culture of intestinal contents. Massive growth of bacteria is accompanied by PCR test for the presence of fimbrial and toxin genes or, for convenience, by agglutination with O-group specific antisera (in Denmark, O8, O45, O64, O138, O139, O141, O149 and O157 specific antisera are used).

Clostridium perfringens type C

Clostridium perfringens (*Cp*) are gram-positive, anaerobic, spore-forming bacteria, which are normal inhabitants of the intestinal tract. *Cp* type C (*Cp*C) are, however, rarely detected in healthy animals (Songer and Uzal, 2005).

Clinical manifestations and relevance

In severe cases, haemorrhagic diarrhoea develops within the first 24 hours of life and piglets rapidly become moribund. Mortality may reach 100% and some piglets die within 12-36 hours of birth without previous signs of diarrhoea. In less severe cases, diarrhoea may be non-haemorrhagic and mortality markedly lower (Songer, 2012).

During the later years, *Cp*C are rare findings in cases of neonatal diarrhoea (Chan et al., 2013; Cruz Jr et al., 2013; Farzan et al., 2013; Gin et al., 2010; Lippke et al., 2011; Svensmark, 2009). In Denmark, routine vaccination of sows pre-farrowing with beta toxoid vaccines is common.

Pathogenesis

CpC mainly colonize in the small intestine, where exotoxins are released into the lumen. Beta-toxin is the essential toxin and action of this toxin produces intestinal necrosis and haemorrhage with loss of absorptive cells leading to excessive accumulation of fluid (Sayeed et al., 2008; Uzal et al., 2010). Protease inhibitors in colostrum and low trypsin production in neonatal pigs prevent the degradation of beta-toxin and contribute to the vulnerability of neonates to disease (Songer, 2012).

Pathology

Predominant gross lesions are seen in the small intestine and include hyperemia, extensive necrosis of mucosa and blood-staining of contents. Microscopically, necrotic intestinal tissues inhabited by bacilli are hallmark lesions (Brown et al., 2007).

Diagnosis

A diagnosis of *Cp*C-related diarrhoea is normally assigned if massive growth of *Cp* is detected by anaerobic culture of intestinal contents and the presence of genes for alfa and beta toxin is verified by PCR.

Viruses

Transmissible gastroenteritis virus

Transmissible gastroenteritis virus (TGEV) is an enveloped, single-stranded RNA-virus in Group 1a of the genus *Coronavirus* in the family *Coronaviridae* (MacLachlan and Duboi, 2011).

Clinical manifestations and relevance

In non-Immune herds TGEV causes epidemic outbreaks of severe diarrhoea in pigs of all ages. Neonatal animals are most severely affected, and clinical signs include vomiting, profuse watery yellow diarrhoea,

severe dehydration and rapid loss of condition. Death occurs within 2-7 days in most or all neonatal piglets, whereas death is uncommon when infection occurs after the age of 2-3 weeks (MacLachlan and Duboi, 2011).

Today, virulent outbreaks of TGE are rare. This is probably due to immunological cross-protection induced by porcine respiratory coronavirus (PRCV) (Miyazaki et al., 2010). PRCV is a deletion mutant of TGEV that has tropism for respiratory tissue, which is enzootic in swineherds worldwide (MacLachlan and Duboi, 2011). This virus was introduced in Europe in the late 1980s (Miyazaki 2010).

Denmark is considered free from TGEV, and the disease is reportable. Serological surveillance of TGEV is carried out in breeding animals destined for export (personal communication, B. Strandbygaard, National Veterinary Institute, Technical University of Denmark).

Pathogenesis

TGEV selectively infects and destroys mature enterocytes lining the intestinal villi, leading to shortening and blunting of villi. The destruction of enterocytes results in malabsorption and maldigestion (MacLachlan and Duboi, 2011).

Pathology

Grossly, intestines are flaccid and distended with fluid contents which may be frothy and with flecks of mucus. Pronounced thinning of the intestinal wall due to loss of villi may be evident, but an unspecific gross appearance is not uncommon (Brown et al., 2007; MacLachlan and Duboi, 2011).

Diagnosis

Mucosal impression smears of intestine can be stained for TGEV by immunofluorescence and antigen ELISAs are available for use in faeces. In order to distinguish between PRCV and TGEV infection, virus-specific RT-PCR assays or competitive ELISA methods must be used (MacLachlan and Duboi, 2011).

Porcine Epidemic Diarrhoea virus

Porcine Epidemic Diarrhoea virus (PEDV) is an enveloped, single-stranded RNA-virus in Group 1b of the genus *Coronavirus* in the family *Coronaviridae* (MacLachlan and Duboi, 2011).

Clinical manifestations and relevance

Outbreaks of PED in suckling piglets are clinically indistinguishable from outbreaks of TGE (Stevenson et al., 2013), though vomiting seems to be less common (MacLachlan and Duboi, 2011). Morbidity in neonatal piglets may reach 100% and mortality averages 50% of piglets in this age group. Spread of PEDV seems to be slower than spread of TGEV (Saif et al., 2012).

PEDV was first recognized in the UK in the 1970s. Outbreaks were subsequently seen in many other European countries like Spain, Italy, The Netherlands, Belgium and The Czech Republic. During the 1980s and 1990s outbreaks in Europe were generally mild and infrequent. However, in 2006 PED re-emerged in epidemic form in Italy (Martelli et al., 2008). Currently, PEDV causes severe outbreaks of diarrhoea in Asia, USA (since April 2013) and Canada (since January 2014) (Anonymous, 2014; Saif et al., 2012; Stevenson et al., 2013).

Denmark is considered free from PEDV, and the disease is reportable. Serological surveillance of PEDV is not carried out routinely (personal communication, B. Strandbygaard, National Veterinary Institute, Technical University of Denmark).

Pathogenesis
Like TGEV, PEDV infects mature enterocytes leading to shortening and blunting of villi.

Pathology
Gross and microscopic lesions resemble lesions in TGE.

Diagnosis
Immunoflourescence staining of intestinal smears or detection of viral RNA in faeces by RT-PCR or ELISA can be used to confirm the diagnosis (MacLachlan and Duboi, 2011).

Rotavirus A
Rotavirus A (RVA) belongs to the genus *Rotavirus* in the family *Reoviridae*, and is a non-enveloped virus with a multi-segmented, double-stranded RNA genome (MacLachlan and Duboi, 2011).

Clinical manifestations and relevance
RVA-related diarrhoea can be seen in piglets from one day of age. Since rotavirus is enzootic in most swineherds, a solid maternal immunity is normally passed on to the piglets and clinical manifestations in neonatal piglets are mild. The duration of diarrhoea in uncomplicated cases is two to three days and mortality is low (Chang et al., 2012).

RVA is among the most prevalent agents in cases of neonatal diarrhoea, and is often detected as the sole infectious agent (Chan et al., 2013; Costinar et al., 2011; Katsuda et al., 2006; Yaeger et al., 2002). However, since the majority of studies are based on qualitative detection of RVA without histologic verification, the causative significance is most often unknown. In a recent study diagnosis of RVA-related enteritis was based upon a combination of histopathology and the detection of viral antigen in enterocytes or in faeces. In this study, RVA-related enteritis was diagnosed in 28 of 237 (12%) submitted neonatal gastrointestinal cases. In one third of these cases, RVA was detected in combination with other agents (Chan et al., 2013).

Pathogenesis
RVA infects and destroys mature enterocytes at the top of intestinal villi, leading to blunting and shortening of villi. These lesions result in malabsorption and maldigestion. Furthermore, the viral enterotoxin NSP4 may also be involved. (MacLachlan and Duboi, 2011).

Pathology
At necropsy, the small intestine is thin-walled, flaccid and dilated with watery contents. Histological lesions include stunting and adhesion of small intestinal villi (Janke et al., 1988; Pearson and McNulty, 1977).

Diagnosis
Impression smears from the small intestine or fixed tissue segments can be subjected to immunohistochemistry for visualization of RVA. The diagnostic value of qualitative identification of RVA in faecal speci-

mens is limited, since RVA is highly prevalent in healthy animals (Gelberg et al., 1991; Svensmark et al., 1989b).

Parasites

Cystoisopora suis

Cystoisospora suis (*C. suis*) is a coccidian parasite (Lindsay et al., 2012) .

Clinical manifestations and relevance

In herd-settings, coccidiosis caused by *C. suis* typically occurs around seven to ten days of age (Lindsay et al., 2012).

Coccidiostats are widely used in modern swine production and therefore coccidiosis has limited clinical importance.

Pathogenesis

C. suis replicate in epithelial cells, mainly in jejunum and ileum. Piglets usually become infected within the first days of life, but clinical signs of diarrhoea do not occur until maximal infection of epithelial cells and accompanying lysis takes place. These events take place on the fourth to fifth day of infection (Brown et al., 2007). Piglets experimentally inoculated with high doses of oocysts have been shown to develop diarrhoea within three days (Stuart et al., 1982). However, in natural cases of coccidiosis, such a short incubation period is unlikely (Brown et al., 2007).

Pathology

Gross lesions in severely affected neonatal piglets include fibrinonecrotic membranes in jejunum and ileum. Microscopically, necrosis of intestinal villi with adherence of fibrinonecrotic cellular debris is reported. In some piglets, no gross lesions are seen and microscopic lesions are limited to atrophy and fusion of villi and erosive changes (Stuart et al., 1982). Endogenous stages of *C. suis* can be detected within epithelial cells in the distal part of intestinal villi (Brown et al., 2007).

Diagnosis

Oocysts in faeces can be demonstrated by flotation methods. Faecal excretion of oocysts fluctuates from day to day. Peak excretion usually occurs two to three days after the onset of clinical signs (Lindsay et al., 2012)

2.3.2 Clostridia of uncertain clinical importance

This section deals with *Clostridium perfringens* type A and *Clostridium difficile*, which both have been incriminated in cases of neonatal diarrhoea. Reviews on experimental and epidemiological studies are followed by evaluations of the scientific basis for associating these bacteria with neonatal diarrhoea in piglets

Clostridium perfringens type A

Clostridium perfringens (*Cp*) are gram-positive, anaerobic, spore-forming bacteria, which are normal inhabitants of the intestinal tract in animals. *Cp* type A (*Cp*A) are commonly detected in healthy animals (Songer and Uzal, 2005).

Experimental inoculations

The first study on the potential significance of *Cp*A in suckling piglet diarrhoea was published in 1983 (Nabuurs et al., 1983). In this study, *Cp*A was detected in 28 of 28 8 to 20-day old diarrhoeic piglets from one herd. A field isolate was subsequently used for inoculation of four 4-day old Hysterectomy Derived Colostrum Deprived (HDCD) piglets. All inoculated piglets developed diarrhoea.

In 1985, (Olubunmi and Taylor) isolated *Cp*A from 12 out of 130 cases of suckling piglet diarrhoea from eight different herds An isolate of *Cp*A obtained from one of these cases was used for experimental inoculation of three 4-day old HDCD piglets. All inoculated animals developed diarrhoea.

One large-scale experimental infection has been published (Johannsen et al., 1993a). In this study, conventional piglets were inoculated prior to uptake of colostrum. The experiment included both inoculation with *Cp*A bacteria and bacteria-free supernatant containing alfa toxin. Approximately one third of the bacteria-inoculated animals developed diarrhoea.

Table 2.1 summarizes methods and results of published experimental inoculations of *Cp*A.

Proposed pathogenesis

Investigations on the potential mechanism of disease have focused upon the excretion of exotoxins. Neonatal piglets are especially vulnerable to such toxins, since trypsin inhibitors in colostrum prevent the degradation within the intestinal contents.

In experimental studies, bacteria were seen adjacent to the epithelium (Olubunmi and Taylor, 1985) or within the luminal contents (Johannsen et al., 1993b). In most subsequent studies, the localization of bacteria was not reported. In a recent case-report, however, the localization of bacteria in close contact with the epithelium and within necrotic epithelial lesions was suggested to indicate causality (Silva et al., 2013).

Experimental studies carried out by Johannsen et al. (1993a) indicated that alfa-toxin is not an important pathogenic factor in *Cp*A-related diarrhoea.

Early studies on *Cp*A-related diarrhoea indicated a pathogenic significance of enterotoxin (Collins et al., 1989; Popoff and Jestin, 1985). In the study by Popoff and Jestin, injection of enterotoxin into ligated intestinal loops resulted in accumulation of fluid in three of three piglets injected with enterotoxin. Collins and co-workers detected enterotoxin in four out of five diarrhoeic neonatal piglets but in none of five

Table 2.1. Summary results of experimental inoculations of *Cp*A bacteria or *Cp*A-derived alfa-toxin.

$N_{inoculated}$	$N_{controls}$	Inoculum	Clinical outcome[1]	Pathology[2]	Reference
4 4-day old HDCD[3] piglets	0	Bacteria ($12*10^9$)	4/4 piglets: yellow pasty or milky faeces within 2-4 days post inoculation	- Villous oedema - Epithelial degeneration at villous tips (with no signs of villous atrophy) - Gas formation throughout the lumen of the intestine. Intramural gas in colon - Inflammation of colonic serosa - Focal necrosis of colonic mucosa	(Nabuurs et al., 1983)
3 4-day old HDCD piglets	3	Bacteria (10^9)	3/3 piglets: Profuse, creamy diarrhoea with flecks of blood within 2 days post inoculation	Villous atrophy Areas with haemorrhages and necrosis in the small intestine	(Olubunmi and Taylor, 1985)
47 1 to 2-hours old CD[4] piglets	17	Bacteria ($3*10^7$ – $3*10^{12}$)	15/47 piglets: Mucoid liquid diarrhoea. A few cases with blood. 5 died	- Mucosal hyperaemia (13%) - Mild enterocolitis (13%) - Haemorrhagic enterocolitis (4%) - Swelling of mesenterial lymph nodes (47%)	(Johannsen et al., 1993a)
23 1 to 6-hours old CD piglets	12	alfa-toxin (80-800 MLD[5])	3/23 piglets: Mucoid liquid diarrhoea	- Mild enterocolitis (26%) - Swelling of mesenterial lymph nodes (26%)	(Johannsen et al., 1993a)

[1]: In all studies, clinical signs were only seen in inoculated animals. [2]: In all studies, pathological lesions were only seen in inoculated animals. Percentages relate to the prevalence within inoculated. [3]: Hysterectomy Derived Colostrum Deprived. [4]: Colostrum Deprived. [5]: Mouse Lethal Doses (the presence of other toxins than alfa-toxin in the supernatant was not thoroughly accounted for).

healthy ones. In another study, *Cp*A-isolates were tested for production of enterotoxin (Estrada Correa and Taylor, 1989) . In this study, five out of 23 isolates from diarrhoeic pigs produced enterotoxin, whereas ten out of ten isolates from non-diarrhoeic pigs did not.

In 1990, Damme-Jongsten et al. tested a total of 112 isolates from both diarrhoeic and healthy piglets of one to three weeks of age, and did not detect enterotoxin genes or production of enterotoxin in any of them. Recent studies supported these findings, since enterotoxin genes were not detected in any *Cp*-isolates from 77 sampled piglets (Cruz Jr et al., 2013; Melin et al., 2010). Altogether, it seems that enterotoxin production is unlikely to be of pathogenic significance in clinical outbreaks.

In 1997, Gibert and co-workers identified beta2-toxin, a toxin that is cytotoxic and lethal to mice, thus resembles beta-toxin produced by *Cp*C (Gibert et al., 1997). *Cp*-isolates from cases of necrotic enteritis in piglets were tested for the presence of genes encoding beta toxin (*cpb*) and beta2 toxin (*cpb-2*). In 12 of 27 isolates (44%) *cpb-2* was the only gene detected. These findings led the authors to suggest that beta2-toxin might be involved in *Cp*A-related enteritis.

Epidemiologic studies

Published studies on epidemiologic associations between *Cp*A and neonatal diarrhoea mainly focus on the detection of *cpb-2* genes. Table 2.2 summarizes the major epidemiological studies on *cpb-2* that included both diarrhoeic and non-diarrhoeic piglets.

Studies on *Cpb-2* gene expression in bacterial isolates from piglets are scarce. One in vitro study found an almost 100% correlation between *Cpb-2* genotype and beta2-toxin expression (Bueschel et al., 2003). In vivo, the situation might be different. In a recent study, *Cpb-2* genes were detected in 93% of *Cp* isolates, irrespective of the diarrhoeal status of the piglet of origin. In the same study, intestinal contents from diarrhoeic (n=24) and non-diarrhoeic (n=8) piglets were tested by ELISA for the presence of beta2-toxin. In both groups, 37.5% of animals were toxin-positive (Farzan et al., 2013). Thus, in natural settings, it seemed that detection of *cpb-2* did not reflect beta2-toxin production. Furthermore, the data of this study, though limited in size, suggested that beta2-toxin was not associated with neonatal diarrhoea. The study by Farzan et al. also included quantification of *Cp*A bacteria in intestinal contents and detected a significantly higher amount of bacteria in non-diarrhoeic vs. diarrhoeic piglets.

Since 2004, detection of *Cp*A by culture has been included in routine diagnostic protocols on neonatal diarrhoea in Denmark. *Cp*A are detected in approximately 80% of submissions and are by far the most prevalent agents detected in laboratory submissions on neonatal diarrhoea at the Laboratory for Swine Diseases in Kjellerup, Denmark.

Sum up on scientific evidence for an association between CpA and neonatal diarrhoea

Despite intensive research for a period of approximately 30 years, scientific evidence of a clinically important association between *Cp*A and neonatal diarrhoea is lacking. Experimental inoculations have shown that high doses of the bacterium can induce diarrhoea and enteral pathology in colostrum-deprived neonatal piglets. However, in natural herd settings, a clinical effect has not been shown, and epidemiologic studies do not link the detection of *Cp*A, *Cpb-2* genes or beta2-toxin with neonatal diarrhoea. It is, however, worth noticing that *Cp* can produce up to 16 different toxins (Uzal et al., 2010) and in principle any of them could have a not yet discovered pathogen potential.

Table 2.2. Summary results of studies on associations between *cpb-2* genes and diarrhoea in suckling piglets.

Study design	Study unit	Prevalence of *cpb-2* in diarrhoeic specimens	Prevalence of *cpb-2* in non-diarrhoeic specimens	Reference
Piglets from 18 Dutch herds with a history of diarrhoea or sudden death were examined	0 to 2-week old piglets	21/30 (70%)	4/14 (29%)	(Klaasen et al., 1999)
Piglets from 51 Swiss herds with a history of diarrhoea or sudden death were examined	0 to 2-week old piglet	28/31 (90%)	16/20 (80%)	(Klaasen et al., 1999)
Screening of *Cp* isolates from different collections and diagnostic laboratories	*Cp* isolates from piglets	235/256 (92%)	305/364[1] (84%)	(Bueschel et al., 2003)
Age-matched diarrhoeic and healthy piglets from 10 herds with a history of neonatal diarrhoea were examined	Cp isolates from 36 diarrhoeic and 12 healthy 1 to 13-day old piglets	147/158 (93%)	40/43 (93%)	(Farzan et al., 2013)
Faecal samples from herd-matched diarrhoeic and healthy pigs from 15 herds were examined	Faecal samples from 1-7 day old piglets	21/30 (70%)	23/30 (76.7%)	(Cruz Jr et al., 2013)

[1]: It was not clearly stated if the 364 isolates included the 256 diarrhoeic isolates.

Clostridium difficile

Clostridium difficile (*Cd*) are gram-positive, anaerobic, spore-forming bacteria. In humans, *Cd* are associated with pseudomembranous colitis, which is primarily seen in elderly, hospitalized and medicated patients. *Cd*-related diarrhoea is recognized as the most common type of nosocomial diarrhoea in humans. In animals, *Cd* are associated with typhlitis in hamsters, necrotizing enterocolitis in neonatal foals and typhlitis in adult horses (reviewed by Songer et al., 2000).

Experimental inoculations

In experimental infections of HDCD piglets, *Cd* induce severe diarrhoea and typhlocolitis, and piglets have been used as animal models for pseudomembranous colitis (Steele et al., 2010; Steele et al., 2013).

Lesions in piglets are mainly restricted to cecum and colon and vary from grossly in-apparent multifocal necrosis to transmural necrosis. Histologically, foci of exudation of mucus, fibrin and aggregates of neutrophils (so-called "volcano-lesions") are seen (Keel and Songer, 2006). Some authors suggest mesocolonic edema to be indicative of *Cd* infection (Cappuccio et al., 2009; Keel and Songer, 2006). However, a recent study suggested mesocolonic edema to be a herd-related phenomenon, rather than an indication of *Cd*-related disease (Cruz Jr et al., 2013). Interestingly, this study only detected mesocolonic edema in piglets from six out of fifteen herds and, on the other hand, detected this finding in four out of four piglets from one of these herds.

Proposed pathogenesis

When studied in laboratory animals, two exotoxins, toxin A and toxin B, are involved in the damage of intestinal epithelial cells associated with *Cd*-related typhlocolitis. For animals to be susceptible to disease, intestinal receptors for toxin A must be present. No specific receptor for toxin B has been identified, and it seems that this toxin causes intestinal damage only if there is a concomitant exposure to toxin A (Keel and Songer, 2006). A binary toxin, *Cd*-transferase has also been suggested to be an important virulence factor in *Cd*-related diarrhoea (Silva et al., 2011).

In a study by Yaeger and co-workers, detection of *Cd* toxin (not distinguishing between toxin A and toxin B) was positively associated with typhlitis and colitis in neonatal piglets, but negatively associated with diarrhoea (Yaeger et al., 2007). Thus, an association between *Cd* toxins A/B and diarrhoea in piglets has not been documented. Studies evaluating the role of binary toxin in neonatal diarrhoea in piglets are not available.

Epidemiologic studies

In a survey on agents in cases of neonatal diarrhoea, *Cd* was detected in 29 of 100 piglets and in 55% of submissions (Yaeger et al., 2002). The survey also included detection of *E. coli*, *Salmonella spp*, *Enterococcus durans*, *C. perfringens*, porcine reproductive and respiratory syndrome virus (PRRSV), rotavirus and TGEV. In 66% of positive piglets, *Cd* was the only pathogen detected. A limited number of studies have compared findings in diarrhoeic and non-diarrhoeic piglets (Alvarez-Perez et al., 2009a; Avbersek et al., 2009; Silva et al., 2011; Yaeger et al., 2007). Avbersek et al. reported that the prevalence of *Cd* as detected by culture in diarrhoeic vs. non-diarrhoeic piglets was not significantly different, but did not go into details on detection rates. Results of the remaining studies are presented in Table 2.3.

Table 2.3. Summary results of studies on associations between the detection of *C. difficile* (*Cd*) and diarrhoea in neonatal piglets.

Study design	Study unit	Prevalence of *Cd*/ *Cd*-toxin/ *Cd* toxin genes in diarrhoeic specimens	Prevalence of *Cd*/ *Cd*-toxin/ *Cd* toxin genes in non-diarrhoeic specimens	Reference
Piglets from 34 herds submitted for laboratory examination with a clinical complaint of diarrhoea and apparently healthy piglets from 5 herds were examined	Contents from jejunum, ileum, proximal and distal colon from 1 to 7-day old piglets evaluated by ELISA[1]	Toxin: 42/100 (42%)	Toxin: 23/29 (79%)	(Yaeger et al., 2007)
Diarrhoeic and non-diarrhoeic piglets from 13 herds were examined	Rectal swabs from 1 to 7-day old piglets evaluated by culture and PCR[2]	Toxin-genes: 58/254 (23%) Culture: 82/254 (32%)	Toxin-genes: 75(287 (26%) Culture: 58/287 (20%)	(Alvarez-Perez et al., 2009a)[3]
2 diarrhoeic and 2 non diarrhoeic piglets from 15 herds were examined	Faecal samples from 1 to 7-day old piglets were evaluated by culture and ELISA	Toxin: 7/30 (23%) Culture: 5/30 (17%)	Toxin: 3/30 (10%) Culture: 7/30 (17%)	(Silva et al., 2011)

[1]: Enzyme Linked Immunosorbent Assay.[2]: Polymerase chain Reaction. [3]: The study detected large differences in prevalence between herds and between geographical regions. However, detection of *Cd* or *Cd* toxins was not associated with diarrhoea within any herd.

In 2008, the prevalence of *Cd* (detected by culture) was investigated in piglets submitted to the Laboratory for Swine Diseases in Kjellerup, Denmark with anamnestic information on neonatal diarrhoea. A positive culture was seen in 23 of 60 (38%) (personal communication, B. Svensmark, DPRC). Detection of *Cd* is not included in routine diagnostic protocols in Denmark.

Sum up on scientific evidence for an association between Cd and neonatal diarrhoea

The main part of research on *Cd* Infection in piglets involves experimental inoculation of HDCD piglets, and is not directly applicable to natural herd situations. However, the experimental inoculations have shown that *Cd* can induce diarrhoea and severe enteral pathology in piglets.

In field cases of neonatal diarrhoea, neither the detection of bacteria nor the detection of *Cd* A/B toxins has been convincingly associated with diarrhoea. More studies focusing on the detection of toxins are needed to confirm or disprove a link with diarrhoea.

2.3.3 Agents with unknown clinical importance in neonatal diarrhoea

To give a clear-cut overview of yet unrecognized agents is of course a contradiction in terms. However, I have tried to provide a summary review of agents that have been studied in such detail – experimentally or epidemiologically – as to suggest a possible association with neonatal diarrhoea in piglets. Most of the epidemiologic studies, however, have not focused on the neonatal piglets, rather evaluated suckling piglets as a whole. At the end of the section I sum up general comments and reflect upon the potential clinical importance of these yet unrecognised agents.

Bacteria

E. coli with non-classical virulence factors

Pathotypes of *E. coli* other than *ETEC* may be associated with neonatal diarrhoea. Non-ETEC virulence factors include Enteroaggregative *E. coli* Heat-Stable Enterotoxin 1 (EAST-1), intimin, Adhesin Involved in Diffuse Adherence (AIDA-I), and Porcine attaching and effacing-associated factor (Paa) (Ngeleka et al., 2003).

EAST-1 is an enterotoxin that resembles STa in terms of size, heat-stability and secretory effect on intestinal cells (Savarino et al., 1991). Intimin, AIDA-I and Paa are non-fimbrial adhesion factors, which seem to be involved in different patterns of adhesion of *E. coli* to epithelial cells (reviewed by Ha et al., 2004).

Experimental inoculations

Experimental inoculations of newborn pigs have been performed using isolates of five different non-classical *E. coli* pathotypes; AIDA-I/STb, AIDA-1/STb/EAST-1, EAST-1, intimin and intimin/EAST-1/Paa (Ngeleka et al., 2003). In these experiments, two piglets were inoculated with each pathotype. Except from the piglets inoculated with the EAST-1 pathotype, which stayed healthy, all piglets developed diarrhoea within 12-20 hours.

Proposed/ suspected pathogenesis

In theory, all *E. coli* isolates being able to create an intimate contact with the intestinal mucosa and produce sufficient amounts of enterotoxin should have a pathogen potential (Brown et al., 2007). Detailed information on the suspected mode of action of EAST-1 and the non-fimbrial adhesion factors is beyond the scope of this review.

Epidemiologic studies

Several studies have identified EAST-1 positive *E. coli* isolates in samples from diarrhoeic suckling piglets (Byun et al., 2013; Chapman et al., 2006; Choi et al., 2001; Ha et al., 2004; Ngeleka et al., 2003; Vu-Khac et al., 2007; Zajacova et al., 2012). In many isolates, EAST-1 is the only virulence factor detected (Choi et al., 2001; Ngeleka et al., 2003). A few epidemiologic studies have included samples from non-diarrhoeic animals (Chapman et al., 2006; Ngeleka et al., 2003; Vu-Khac et al., 2007; Zajacova et al., 2012). Ngeleka et al. detected EAST-1 genes in 27% (46/170) of isolates from diarrhoeic piglets vs. 14% (17/120) of isolates from non-diarrhoeic piglets (for convenience, isolates from diarrhoeic vs. non-diarrhoeic piglets are referred to as diarrhoeic vs. non-diarrhoeic isolates in the following). Vu-Khac et al. detected EAST-1 genes in 65% (144/220) of diarrhoeic isolates vs. 27% (8/30) of non-diarrhoeic isolates. In the remaining studies the prevalence of EAST-1 genes was the same in diarrhoeic vs. non-diarrhoeic isolates.

The prevalence of intimin positive *E. coli* in diarrhoeic vs. non-diarrhoeic suckling piglets has been evaluated in a few studies (Martins et al., 2000; Ngeleka et al., 2003; Vu-Khac et al., 2007). Martins et al. detected intimin genes in 25.7% of diarrhoeic vs. 9.5% of non-diarrhoeic isolates. Vu-Khac et al. detected intimin genes in 56% (124/220) of diarrhoeic vs. 17% (5/30) non-diarrhoeic isolates. An opposite trend was seen in the study by Ngeleka et al., which detected intimin genes in 2% of diarrhoeic vs. 15% of non-diarrhoeic isolates. In a recent Korean study only involving diarrhoeic suckling piglets intimin genes were not detected in any of 116 *E.coli* isolates (Byun et al., 2013).

AIDA-I was detected in 2-10% of diarrhoeic isolates in different studies (Byun et al., 2013; Chapman et al., 2006; Ha et al., 2004; Ngeleka et al., 2003). In two studies including non-diarrhoeic isolates, AIDA-I was not detected in those (Chapman et al., 2006; Ngeleka et al., 2003).

Paa was detected in 29% (34/116) of diarrhoeic isolates from suckling piglets in 110 Korean pig herds (Byun et al., 2013). A previous study detected Paa genes in 9% (15/170) of diarrhoeic and 8% (9/120) of non-diarrhoeic isolates from suckling piglets in Canada (Ngeleka et al., 2003).

Enterococcus

The genus *Enterococcus* consists of gram-positive, facultative anaerobic bacteria, which are an essential part of the intestinal microbiota in humans and animals. They belong to the group of lactic acid bacteria and are used as probiotics due to their production of bacteriocins. The *Enterococcus faecium* group (comprising *Enterococcus faecium*, *Enterococcus durans*, *Enterococcus hirae, Enterococcus mundtii*, *Enterococcus porcinus* and *Enterococcus villorum*) is the most prevalent in production animals. The distribution of *Enterococcus* species varies between countries. In Denmark, *Enterococcus hirae* is the dominant species isolated from animals (Fisher and Phillips, 2009).

Experimental inoculations

Experimental inoculations of suspected pathogenic strains of *Enterococcus spp.* have not been conducted.

Proposed/ suspected pathogenesis

The ability of *Enterococcus spp.* to adhere to the mucosal surface and cause disease seems to be associated with virulence factors such as Aggregation substance, Extracellular surface protein, Endocarditis- and biofilm-associated pili, cytolysin and hydrolytic enzymes (Fisher and Phillips, 2009; Vancanneyt et al., 2001).

Epidemiologic studies

A few studies suggest *Enterococcus* spp. to be associated with neonatal diarrhoea (Cheon and Chae, 1996; Larsson et al., 2013). In the study by Cheon and co-workers, *Enterococcus spp* were associated with an outbreak of diarrhoea with high morbidity in 2 to 14-day old piglets. Isolates from this outbreak were subsequently identified as *Enterococcus villorum* (Vancanneyt et al., 2001). Larsson and co-workers identified *Enterococcus spp.* in 18 of 50 (36%) diarrhoeic neonatal piglets and in none of 20 non-diarrhoeic piglets. Both of the mentioned studies reported on extensive colonization of the small intestinal mucosa by *Enterococcus spp.*

Viruses

Rotavirus B

Rotavirus B (RVB) belongs to the genus *Rotavirus* in the family *Reoviridae,* and is a non-enveloped virus with a multi-segmented, double-stranded RNA genome (MacLachlan and Duboi, 2011).

Experimental inoculations

In an experimental study, RVB induced diarrhoea and/ or caused histopathological lesions in 11 out of 11 five to six day-old HDCD piglets. The virus-isolate was originally obtained from a diarrhoeic 27-day-old piglet. Intestinal lesions were similar to but less extensive than lesions caused by RVA and seemed to repair rather quickly (Theil et al., 1985).

Proposed/ suspected pathogenesis

Experiments have shown that the pathogenesis in RVB-infection is similar to RVA-infection (Theil et al., 1985).

Epidemiologic studies

RVB can be detected in suckling pigs with diarrhoea, but epidemiologic studies suggest a relatively low prevalence (Janke et al., 1990; Katsuda et al., 2006; Marthaler et al., 2012; Medici et al., 2011). It seems that infection with RVB is most often accompanied by infection with other rotaviruses.

Rotavirus C

Rotavirus C (RVC) belongs to the genus *Rotavirus* in the family *Reoviridae*, and is a non-enveloped virus with a multi-segmented, double-stranded RNA genome (MacLachlan and Duboi, 2011).

Experimental inoculations

RVC has been associated with a well-described herd-outbreak of neonatal diarrhoea (Morin et al., 1990). Intestinal contents from piglets diagnosed with RVC-infection were filtered and subsequently the bacteria-free filtrate was used as inoculum for seven 2-day-old colostrum deprived piglets. Five out of seven inoculated piglets developed villous atrophy and some (number not specified) developed yellow, liquid faeces.

Proposed/ suspected pathogenesis

Experimental inoculations indicated a pathogenesis like in RVA-infection (Morin et al., 1990).

Epidemiologic studies

Recent studies demonstrated a high prevalence of RVC in diarrhoeic suckling pigs (Amimo et al., 2013; Marthaler et al., 2012). In the study by Marthaler et al., 50% of samples from diarrhoeic piglets below three

weeks of age were RVC positive, and in 74% of these, RVC was the only rotavirus group detected. Amimo and co-workers detected RVC in 51% (n=34) of diarrhoeic vs. 16% (n=10) of non-diarrhoeic samples. In another study, RVC was the only rotavirus detected in 78% (n=442) of rotavirus-positive samples in 1-3 day-old diarrhoeic piglets (Marthaler et al., 2013). Since histological lesions (denuded villous tips resulting from enterocyte necrosis and sloughing) were also seen in positive piglets the finding might imply that RVC was causatively associated with diarrhoea. However, neither of the studies reported on the detection of other pathogens and herd-associations were not considered (almost all non-diarrhoeic samples in the study by Amimo and co-workers came from the same herd).

Porcine adenovirus

Adenoviruses are non-enveloped viruses with a double-stranded DNA genome in the genus *Mastadenovirus* of the family *Adenoviridae*. Porcine adenovirus has been associated with respiratory, enteric and central nervous infections, but is generally regarded as a rare cause of disease in pigs (MacLachlan and Duboi, 2011).

Experimental inoculations

Diarrhoea has been experimentally induced by inoculation of 2 day-old piglets (Ducatelle et al., 1982). In this study, piglets developed diarrhoea after an incubation period of one to four days.

Proposed/ suspected pathogenesis

In the experiment by Ducatelle and co-workers, histological evidence of short and blunt villi suggested malabsorption and maldigestion to be the pathogenic mechanism in diarrhoea (Ducatelle et al., 1982).

Epidemiologic studies

Porcine adenovirus has been incriminated in herd outbreaks of suckling piglet diarrhoea in Canada and USA. In three American herd outbreaks supplement diagnostics did not detect other aetiological explanations on the diarrhoea (Abid et al., 1984). However, combining results from two large American and Canadian surveys, porcine adenovirus was detected as the sole agent in only eight out of 1728 (0.5%) cases of piglet diarrhoea (Morin et al., 1983; Sanford and Hoover, 1983), thus did not seem to play an important role.

Porcine astrovirus

Astroviruses are non-enveloped single-stranded RNA-viruses in the genus *Mamastrovirus* of the family *Astroviridae*. Astroviruses are ubiquitous in young animals. In turkeys, astroviruses are associated with enteritis and stunting (MacLachlan and Duboi, 2011).

Experimental inoculations

Experimental inoculation of three 4-day old gnotobiotic piglets with porcine astrovirus resulted in mild diarrhoea (Shimizu et al., 1990).

Proposed/ suspected pathogenesis

The pathogenesis in enteric astrovirus infections has been studied in turkeys, but is still not clarified. Astroviruses do not cause villous atrophy, but appear to induce maldigestion and may have a direct effect on the integrity of the epithelial barrier (MacLachlan and Duboi, 2011).

Epidemiologic studies

In a survey involving 122 diarrhoeic suckling piglets submitted for laboratory examination, porcine astrovirus was detected in 64% (Mor et al., 2012). However, in 80% of the astrovirus positive piglets (all age groups combined) other viral pathogens were also detected. Any presence of bacteria or parasites was not reported.

Porcine sapovirus

Sapoviruses are non-enveloped single-stranded RNA viruses in the genus *Sapovirus* of the family *Caliciviridae* (MacLachlan and Duboi, 2011).

Experimental inoculations

In two inoculation studies using 4 to 6-day old HDCD piglets, 27 of 27 inoculated piglets developed a mild to moderate diarrhoea (Flynn et al., 1988; Guo et al., 2001). The isolate used in the study by Guo was originally detected in a natural case of diarrhoea in a 27 day-old piglet (Saif et al., 1980).

Proposed/ suspected pathogenesis

The decreased length of intestinal villi seen in experimental infections suggests a malapsorptive pathogenesis (Flynn et al., 1988).

Epidemiologic studies

In a cross-national survey from 2010, porcine sapovirus was detected in 3.3%, 13.3% and 35% of suckling piglets from Hungary, Spain and Slovenia. Danish results were reported as a combined prevalence in suckling and weaned pigs (2-8 weeks of age) and reached 66.7% (Reuter et al., 2010b). Most of the countries in the study only sampled healthy pigs, but Denmark and Spain included diarrhoeic pigs. Samples from these two countries showed no association between diarrhoea and detection of porcine sapovirus (all age-groups combined). In a recent Chinese study, the seroprevalence of porcine sapovirus in diarrhoeic suckling piglets was 62% (594/960 piglets from ten geographical locations). However, virus was only detected in 7% (7/101) of diarrhoeic faecal samples, and the authors concluded that other factors seemed to cause the majority of diarrhoeal problems in that study (Liu et al., 2014).

Porcine norovirus

Noroviruses are non-enveloped single-stranded RNA viruses in the genus *Norovirus* of the family *Caliciviridae* (MacLachlan and Duboi, 2011).

Experimental inoculations

Experimental inoculations of HDCD pigs with noroviruses have been conducted using porcine and human isolates (Cheetham et al., 2006; Wang et al., 2005). In the study using a human isolate, 48 of 65 inoculated pigs (74%) developed mild diarrhoea. Age at inoculation was not reported. In the other study two gnotobiotic pigs were inoculated with a porcine strain of norovirus at the age of 9 days and 35 days, respectively. Both pigs developed mild diarrhoea (Wang et al., 2005).

Proposed/ suspected pathogenesis

Moderate villous atrophy was detected in only one out of seven piglets inoculated with a human strain of norovirus (Cheetham et al., 2006). The authors speculated that the absence of pathological lesions may be

explained by cells dying from apoptosis instead of lysis. However, the pathogenic mechanism remains to be explained.

Epidemiologic studies

Epidemiologic studies indicate that noroviruses are low prevalent in pigs (Park et al., 2010; van der Poel et al., 2000; Wang et al., 2005). Studies conducted in USA and Spain detected noroviruses in approximately 2% of adult pigs, whereas Park and co-workers did not detect noroviruses in any of 84 diarrhoeic pigs from different age groups in Korea.

Porcine kobuvirus

Kobuviruses are non-enveloped single-stranded RNA viruses in the genus *Kobuvirus* of the family *Picornaviridae* (MacLachlan and Duboi, 2011).

Experimental inoculations

No experimental inoculations wit porcine kobuvirus have been performed.

Proposed/ suspected pathogenesis

Kobuviruses are thought to infect the gastrointestinal tract, but the site of replication, replication cycle and target cells are to be determined (Reuter et al., 2011).

Epidemiologic studies

In 2009-2010, Reuter and co-workers tested healthy pigs in the same herd on two occasions separated by 21 months. On both occasions, porcine kobuvirus was detected at a high prevalence in all age groups of pigs (Reuter et al., 2009; Reuter et al., 2010a). The authors concluded that the virus seemed to be endemically circulating and non-pathogen within this herd.

Studies from Asian countries and USA show that porcine kobuvirus is acommon finding in samples from pigs, and that the prevalence seems to be higher in young animals (An et al., 2011; Khamrin et al., 2009; Khamrin et al., 2010; Park et al., 2010; Verma et al., 2013; Wang et al., 2011; Yu et al., 2009).

A few studies included samples from both diarrhoeic and non-diarrhoeic pigs (Park et al., 2010; Wang et al., 2011). Both of these studies detected a higher prevalence of porcine kobuvirus in the diarrhoeic pigs. Park et al. presented the detection rates in different age-groups, and in this study 45 of 46 (97.8%) diarrhoeic suckling piglets vs. 6 of 15 (40%) of non-diarrhoeic piglets were positive. In this study co-infections with other pathogens were detected in 81% of kobuvirus positive diarrhoeic pigs. Wang et al. did not evaluate the presence of other pathogens. Interestingly, another study detected a markedly higher rate of infection in non-diarrhoeic vs. diarrhoeic 20 to 30-day old piglets (Shan et al., 2011). Thus, it is not clear whether kobuvirus is simply a part of the resident microbiota or – perhaps in some cases – has a causative significance in diarrhoea.

Porcine teschovirus and porcine sapelovirus

These viruses belonging to the family *Picornaviridae* have frequently been isolated from diarrhoeic faeces in pigs (Alexandersen et al., 2012). The potential association with neonatal diarrhoea and these viruses has not been investigated.

Parasites

Strongyloides ransomi

Strongyloides ransomi is an intestinal nematode with both parasitic and free-living reproductive cycles. The parasite is ubiquitous in pigs, but so far, a pathogenic significance has only been recognised in suckling pigs in tropical climates. In these cases, infection has been associated with poor weight gain, diarrhoea and increased mortality (Greve, 2012).

Experimental inoculations

Both prenatal and postnatal infections have been experimentally documented (Stewart et al., 1976) but the principal route of infection for neonatal piglets is considered to be transcolostral uptake of larvae (Greve, 2012).

Proposed/ suspected pathogenesis

Strongyloides larvae typically infect the proximal small intestine and establish in tunnels of the epithelium about the base of villi or in upper crypts. Adult worms persist in these tunnels and if in sufficient numbers their presence induces villous atrophy. Thus, the infection can result in malabsorption diarrhoea (Brown et al., 2007).

Epidemiologic studies

The prevalence of *Strongyloides* infection under northwest European housing conditions have been investigated in several studies (Eysker et al., 1994; Haugegaard, 2010; Roepstorff and Jorsal, 1989; Roepstorff et al., 1998). These studies were carried out in 105, 35, 404 and 79 sowherds, respectively. *Strongyloides* eggs were not detected except in the study from 1998, where a prevalence of 0.9, 4.4 and 0.1 was detected in Danish, Icelandic and Swedish lactating sows, respectively. In a study comparing the prevalence in differently managed herds, *Strongyloides ransomi* was not detected in any of four intensively managed herds and in only 2-7% of samples from four extensively managed herds (Roepstorff, 1991).

Cryptosporidium

Cryptosporidium is an obligate intracellular protozoan parasite, with a worldwide distribution in humans and animals (Lindsay et al., 2012). *Cryptosporidium parvum* (*C. parvum*) is a well-known pathogen in young calves, causing watery diarrhoea with high morbidity (Santin, 2013). In humans, *Cryptosporidium spp* are commonly associated with diarrhoea in immune-compromised patients (Feasey et al., 2011). Two species; *Cryptosporidium suis* (*C. suis*) and *Cryptosporidium* pig genotype II (*C. pigII*) are commonly identified in pigs (Lindsay et al., 2012). *C. parvum* is occasionally detected in suckling piglets, but appears to be rare under Danish conditions (personal communication, Heidi Enemark, National Veterinary Institute, Technical University of Denmark).

Experimental inoculations

Experimentally, four out of nine 2 day-old conventional piglets inoculated with *C. suis* oocysts developed mild diarrhoea after a period of two to sixteen days. Co-infection with rotavirus markedly aggravated the severity of clinical symptoms. (Enemark et al., 2003).

Recent attempts to experimentally infect 4-week old piglets with *C. pigII* failed (Kvac et al., 2013). In the same study, 8-week old piglets were successfully infected, but did not show any signs of disease and did not exhibit intestinal lesions.

Proposed/ suspected pathogenesis

Diarrhoea seems to be due to malabsorption associated with villous atrophy and a population of immature enterocytes. Also the occupation of a large proportion of the surface area of intestinal cells by cryptosporidiae, release of inflammatory mediators and an increase in epithelial cell permeability may be important mechanisms of *Cryptosporidium*-associated diarrhoea (Brown et al., 2007).

Epidemiologic studies

C. suis as well as *C. pigII* have been detected in suckling pigs (Hamnes et al., 2007; Jenikova et al., 2011; Kvac et al., 2009; Langkjaer et al., 2007; Vitovec et al., 2006). Two of these studies focused on the age-related detection within single herds (only one herd was included in each of the studies) and did not detect *C. pigII* in any pigs below the age of 6-7 weeks (Jenikova et al., 2011; Kvac et al., 2009). Thus, *C. suis* might be the only relevant species in the youngest pigs. In two large studies conducted in Danish and Norwegian herds, the prevalence of oocyst shedding in suckling piglets was low (Hamnes et al., 2007; MaddoxHyttel et al., 2006). In the Danish study 6% (29/488) of piglets and 31% (16/50) of herds were detected positive and in the Norwegian study 8% (55/684) of litters and 31% (31/100) of herds were positive. *C. parvum* was not detected in any of the five mentioned studies.

One study reported on an association between diarrhoea and the detection of *Cryptosporidium* oocysts (Hamnes et al., 2007). In this study, including litters from 100 herds, 36% (13/36) of diarrhoeic vs. 19% (81/413) of non-diarrhoeic litters were Cryptosporidium positive. *Cystoisospora* was excluded as a cause of diarrhoea in these litters, however, no further aetiological examinations were performed.

Two surveys on enteropathogens in diarrhoeic suckling piglets included the detection of *Cryptosporidium spp.* (de la Fé Rodríguez et al., 2013; Wieler et al., 2001). In the German study, oocysts were detected in 1.4% (4/287) of piglets, whereas the Cuban study reported on the detection of *C. parvum* antigen in 16% (7/45) of piglets.

Giardia

Giardia duodenalis are flagellate protozoans which inhabit the intestine of humans and animals. Seven assemblages (A-G) of *Giardia duodenalis* have been described. In production animals, the host-specific assemblage E and the zoonotic assemblages A and B have been detected. *Giardia* is recognized as the most common parasitological cause of diarrhoea in humans, whereas the clinical importance in production animals is questionable (Geurden et al., 2010).

Experimental inoculations

No experimental infections have been conducted in piglets. Diarrhoea has been experimentally induced in goat kids, lambs and calves by inoculation of *Giardia* cysts (Geurden et al., 2010).

Proposed/ suspected pathogenesis

Giardia trophozoites attach to epithelial cells between villi or in folds on the villous surface in the proximal small intestine. The pathogenesis of giardiasis is considered as a multifactorial process involving microvillous alterations, excretion of toxic products and host immune responses which in combination lead to malabsorptive diarrhoea (Brown et al., 2007; Geurden et al., 2010).

Epidemiologic studies

Large epidemiologic studies conducted in Denmark and Norway detected a low prevalence of Giardia cysts in suckling pigs (3% of piglets vs. 1.5% of litters and 22% of herds vs. 10% of herds, in the respective studies) (Hamnes et al., 2007; MaddoxHyttel et al., 2006). Potential associations with diarrhoea in suckling piglets were only evaluated in the Norwegian study which did not detect any association.

Microsporidia

Microsporidia are obligate intracellular fungi commonly associated with chronic diarrhoea and wasting in immune-compromised human patients. *Enterocytozoon bieneusi* (*E. bieneusi*), *Encephalitozoon intestinalis* and *Encephalitozoon cuniculi* genotype III have been detected in pigs (Lindsay et al., 2012; Valencakova et al., 2006).

Experimental inoculations

Pigs have been experimentally inoculated with *E. bieneusi* spores of human and macaque origin (Kondova et al., 1998; Lvarado G et al., 2009). In these studies, neither immunosuppressed HDCD 1-day old piglets nor one month old piglets developed diarrhoea, though infection was successful in all inoculated pigs. However, Snowden and co-workers reported that immune-suppressed HDCD piglets did develop diarrhoea when inoculated with *E. bieneusi* (unpublished study, cited in Snowden et al., 1998).

Encephalitozoon spp have not been thoroughly investigated in piglet models, but appear to be able to infect a wide variety of cells thus producing disseminated disease (Snowden et al., 1998).

Proposed/ suspected pathogenesis

Experimental infection with *E. bieneusi* resulted in villous atrophy, suggesting a malabsorptive pathogenesis (Lvarado G et al., 2009). *Encephalitozoon* species induce necrosis in both enterocytes and other cell types (Didier et al., 2000).

Epidemiologic studies

In a study involving 77 diarrhoeic and 50 non-diarrhoeic piglets under two weeks of age, *E. bieneusi* was detected in 12% in the diarrhoeic group vs. none in the non-diarrhoeic group (Jeong et al., 2007). No other enteric pathogens were evaluated in that study.

Balantidium coli

Balantidium coli is a ciliated protozoon detected in intestines from pigs and humans (Lindsay et al., 2012). In pigs, the infection is generally considered to be asymptomatic, whereas humans may develop dysentery-like symptoms when infected. The infection in humans is primarily of clinical importance in developing countries (Schuster and RamirezAvila, 2008).

Experimental inoculations

Balantidium coli derived from diarrhoeic humans induced diarrhoea in ten out of ten conventional 2-month-old piglets after experimental inoculation (Yang et al., 1995).

Proposed/ suspected pathogenesis

Histopathological lesions in experimentally inoculated pigs were confined to the mucosa of the distal intestine. Mucosal lesions included necrosis and ulceration. Thus, the pathogenesis of *Balantidium*-associated diarrhoea seems to be loss of absorptive capacity in the large intestine.

Epidemiologic studies

A Danish study conducted in a single herd detected a prevalence of 57% of *Balantidium coli* in healthy suckling pigs (Hindsbo et al., 2000). Almost 100% of piglets above four weeks of age were detected positive.

General sum up on unrecognised pathogens

A commonly used method to investigate potential pathogens has been to sample piglets from a lot of different herds and evaluate associations between detection of the pathogen and the presence of diarrhoea within these piglets. Most studies do not take herd variations into account. However, as exemplified by studies on *C. difficile*, *Cryptosporidium*, *Giardia* and sapovirus (Alvarez-Perez et al., 2009b; MaddoxHyttel et al., 2006; Reuter et al., 2010b) inter-herd (and inter-national) variations are considerable and need to be taken into account when drawing conclusions on the association between a certain pathogen and diarrhoea. Studies that do not take this into account are likely to either over-interpret or overlook associations. Also, when investigating enteral pathogens, it is probably of outmost importance to select piglets from the very specific age group in question. Many studies combine microbiological results from piglets aged 1-30 days, thus do not take the dynamic changes in microbiota into account.

Studies on unrecognised pathogens designed to look into a possible association with neonatal diarrhoea are scarce. However, studies on *Enterococcus spp* (Cheon and Chae, 1996; Larsson et al., 2013) and RVC (Morin et al., 1990) do have this specific approach and do suggest an association. *E. coli* with non-classical virulence factors (probably especially EAST-1 and intimin) might also be important, since several studies show a higher prevalence in diarrhoeic vs. non-diarrhoeic piglets.

Parasites like *Giardia spp*, *Cryptosporidium spp* and *Strongyloides ransomi* seem to be very low prevalent in sows and suckling piglets, thus probably of minor clinical importance in conventional northern European piglets. With respect to microsporidia and *Balantidium coli,* experience from human cases suggests that these agents seldom play a primary role in diarrhoea. The high prevalence of *Balantidium coli* in healthy piglets supports this conclusion.

Studies on rotavirus B, rotavirus C, porcine adenovirus, porcine astrovirus, porcine norovirus, porcine kobuvirus, porcine teschovirus and porcine sapelovirus generally confirm the presence of these viruses in suckling piglets. Studies associating these viruses with diarrhoea most often do not take the presence of other pathogens into account or, when they do, detect a lot of co-infections. Thus, the clinical importance of these viral infections is questionable.

Photo: Martin Dam Kristensen

3 Methods in the project

3.1 Selection of herds

Since, strictly speaking, anecdotal evidence on "something new happening" was the only starting point of the study, the selection of representative study-herds posed a main challenge. In the selection of herds, the main goal was to avoid herds having problems related to well-known agents, immune suppression due to PRRS or obvious management issues. Also, for practical reasons, a relatively high prevalence of diarrhoea and not too much variation between farrowing batches were necessary inclusion criteria.

All veterinary practitioners in the e-mail database of DPRC were invited to recommend suitable herds. The inviting e-mail is enclosed as Appendix 1. In total, 34 herds were recommended by 19 different veterinarians. Herds considered suitable based upon telephone interviews with veterinarians, herd managers and feed consultants, were asked to account for the number of diarrhoeic litters within three consecutive farrowing batches. Only herds with a total litter prevalence around 30% or gilt-litter prevalence around 90% persistently across batches were considered useful for the study.

Five herds fulfilled the requirements. These herds were visited for personal interviews, evaluation of production systems and for sampling of material for a preliminary screening of agents. In each herd, five 1-4-day old piglets from different litters with acute signs of diarrhoea were euthanized. Intestinal contents from these piglets were tested using routine laboratory protocols for *E. coli*, *Clostridium perfringens* type C and rotavirus A. Herds were excluded if *Clostridium perfringens* type C or haemolytic *E. coli* were detected in any piglet or if moderate to massive growth of non-haemolytic *E. coli* of sero-groups O8, O45, O64, O138, O139, O141, O149 or O157 or rotavirus A were detected in more than one piglet. In herds that were not free from PRRS by declaration, 10 blood-samples from affected piglets were tested for PRRS by PCR or antibody ELISA. Herds were excluded if any of the ten samples were positive.

A total of four herds ended up being included in the research programme, since the practical setup in the initially investigated herd was not completely comparable with the setup in the other herds.

3.2 Study designs and priorities

The main priority of the research programme was a case-control investigation involving all the different fields of research (necropsy, histopathology, bacteriology, virology and molecular characterisation using gut-microbiota assays) included in the three Phd-projects.

In parallel with the case-control study, a cross-sectional study with follow up was carried out in order to obtain an insight into the epidemiology of the syndrome.

3.3 Inclusion of sows and piglets and treatments during the study

On three major farrowing days of one farrowing batch per herd, a total of approximately 20 newly farrowed sows were included. On each day, six to eight sows having farrowed during the night or being the first to finish farrowing during the morning were selected. Sows with less than twelve functional teats, having chronic mastitis, leg problems (indolent hoof lesions were accepted) or a known tendency to develop shoulder wounds were not included. In herds predominantly experiencing problems in first parity litters (Herd 2 and 4), first parity sows were given high priority in the inclusion procedure. Since first parity sows were less synchronized, the inclusion of sows in these herds lasted for more than three days, and fewer sows were included per day.

Using lists of random numbers, a total of 11 (Herd 1 and 3) or 12 (Herd 2 and 4), were kept in their original litters (sows with litter sizes smaller than this were not included). The decision to keep 11 or 12 piglets per litter was based upon individual herd-experience on the capacity of sows. Remaining piglets were placed in litters not participating in the study. Only piglets considered viable and with a birth weight above 800g were included.

During the study period, no cross-fostering was allowed. Medication of sows was allowed when considered necessary by the individual herd-managers and consisted of antibiotics and NSAIDs in combination. Piglets were not allowed to be treated with antibiotics prior to day 3 of life (prior to the first day of selection for necropsy – see section 3.7). However, due to ethical considerations, after this day antibiotic treatments were allowed when considered necessary by the herd-managers. As a consequence, the vast majority of piglets being diarrhoeic after day 3 of life were treated with antibiotics. An exception to the rule was made in Herd 4, where a total of 13 piglets were treated with streptocillin due to arthritis prior to day 3. These piglets were not used in the case-control study. Non-antibiotic oral supplements were allowed during the whole study-period, but were only used in a very limited number of piglets in Herds 2 and 4.

3.4 Exclusion of piglets and litters during the study period

If piglets seriously failed to thrive due to other reasons than diarrhoea, they were excluded from the study. Whole litters were excluded if the sow died, developed shoulder ulcers or stopped eating and milking during the study period. If litter size was reduced to less than five piglets the whole litter was excluded.

3.5 Clinical recordings in sows

On the day of parturition, sows were given a body condition score from 1 (skinny) to 4 (fat) and the clinical parameters given in Table 3.1 were registered.

Table 3.1. Clinical recordings in sows on the day of parturition.

Parameter	Interpretation
Rectal temperature	A rectal temperature ≥ 39.5 °C was considered as fever
Mastitis	One or more udder sections were hard, red or sore on palpation
Abnormal vulva discharge	An excess of unclear or foul-smelling discharge
Leg problems	Unwillingness to bear weight on all legs or soreness on palpation

3.6 Clinical recordings in piglets

Piglets were weighted at birth (day one), at ten days of life and at weaning. Clinical examinations were carried out with daily intervals, starting at day one and ending on day five to seven (depending on the timing of clinical signs within the separate herds). The appearance of faeces was determined by use of rectal swabs. Liquid and watery consistencies were considered diarrhoeic. Rectal swabs were stored on dry ice in the herds (in PBS mixed with glycerol) until transportation to the laboratory, where they were stored on -80°C until further use. Table 3.2 presents the clinical parameters registered.

Table 3.2. Clinical recordings in piglets on day 1-5.

Parameter	Categorization
Hollow flanks	Registered if the area behind ribs deviated inwards
Skinny	Registered if ribs and spine were visible
Dull hair coat	Registered if hair coat appeared dull and longer than normal
Dehydration	Registered if a lack of skin-elasticity and sunken eye-balls was noted
Abrasions on fore-knees	Any abrasion
Perineal staining	Registered if staining was seen within a 1 cm diameter around anus
Faecal consistency	Empty rectum
	Watery[1]
	Liquid[1]
	Creamy[2]
	Firm[2]
	Lumpy[2]
Faecal colour	White
	Yellow
	Black
	Green
Faecal admixtures	Blood
	Foam
	Mucous
	Debris

[1]: Piglets with these faecal consistencies were considered diarrhoeic.

[2]: Piglets with these faecal consistencies were considered non-diarrhoeic.

3.7 Selection of Case and Control piglets for necropsy

It was considered important to examine piglets in both early and late phases of disease. Therefore, individual herd-plans on when to euthanize piglets were constructed, taking previous experience on the timing of symptoms into account. In herd 1 and 4, piglets were selected at 3 and 5 days of age. In herd 2, piglets were selected at 4, 5 and 7 days of age and in herd 3, piglets were selected at 4 and 6 days of age.

Case-definition: Piglets which had been diarrhoeic for at least two subsequent days (including the day of selection). Case-piglets were selected within litters having the highest prevalence of diarrhoea on the day of selection.

Control-definition: Piglets with no history of diarrhoea. Control-piglets were selected from litters having the lowest prevalence or no diarrhoea on the day of selection.

A maximum of two piglets per litter was selected for necropsy. Case and control piglets were allways selected from different litters.

3.8 Necropsy and histopathology

Piglets were brought live to the laboratory and euthanized within six hours of selection. Tissue-samples for histological examination were fixed in formalin immediately after euthanasia. Tissue-samples and intestinal contents for virological examination, toxin determination and gut-microbiota assays were snap-frozen on dry ice and stored at -80 °C.

All organs were examined grossly with special attention towards intestinal findings. Histological evaluation was carried out on samples from duodenum, jejunum, ileum and spiral colon.

3.9 Detection of infectious agents

3.9.1 Bacterial culture

Sections of jejunum and colon were cultured aerobically for the detection of *E. coli* and anaerobically for the detection of *C. perfringens* and *C. difficile*. A 24 hour incubation period was used for the detection of *E. coli* and *C. perfringens*, whereas 48 hours of Incubation was used in the detection of *C. difficile*. In all cases the incubation temperature was 37°C.

A piglet was considered *E. coli* positive if any growth of haemolytic colonies or moderate to massive growth of non-haemolytic colonies morphologically resembling *E. coli* was seen on blood agar plates (Columbia agar (Oxoid) supplemented with 5% calf blood) in any section of intestine. Colonies were verified using an in house selective and indicative medium for coliforms. A piglet was considered *C. perfringens* positive if moderate to massive growth of colonies morphologically resembling *C. perfringens* was detected in any section of intestine. Colonies were verified using Tryptose-Sulfite-Cycloserine agar (Oxoid). A piglet was considered *C. difficile* positive if any growth of yellow colonies with a characteristic horse-stable odour was seen on Cycloserine Cefoxitin Fructose agar in any section of intestine.

E. coli isolates (one isolate per *E. coli* positive piglet) were further characterized by O-serogrouping and virulence gene detection by PCR as described by Frydendahl (2002) and Zhang et al. (2007). Table 3.3 lists O-groups and virulence genes that were investigated in the study. Typing of *C. perfringens* by PCR was done on a pool of four isolates per piglet using the methods described by Baums et al. (2004).

Table 3.3. O-groups and virulence-genes investigated in *E.coli* isolates of the study.

O-groups	O8, O45, O64, O138, O139, O141, O149, O157
Adhesin-genes	F4, F5, F6, F18, F41, intimin, Paa, AIDA-1
Toxin-genes	STa, STb, LT, VT2e, EAST-1

3.9.2 Bacterial detection using Flourescence In Situ Hybridization (FISH)

Tissue specimens from duodenum, jejunum, ileum and colon were evaluated by FISH for the presence of *E. coli*, *C. perfringens*, *C. difficile* and *Enterococcus spp*. FISH was carried out with oligonucleotide probes targeting bacterial 16S rRNA and was performed as double hybridization with differently labeled probes; a universal probe targeting *Domain bacteria* and specific probes directed against the listed bacteria. Methods are described in Jonach et al. (2014).

When unspecific bacteria were recognised within necrotic lesions (using the universal probe) a probe targeting *Fusobacterium necroforum* (*F. necroforum*) was used.

3.9.3 Detection of *Cp* beta2-toxin using Enzyme-linked Immunosorbent Assay (ELISA)

The presence of beta2-toxin was detected in contents from jejunum using an in-house sandwich ELISA. Microtitre plates (NUNC, 442404) were coated with beta2 specific monoclonal antibody (3H4; kindly provided by B. Kadra, CEVA-Phylaxia, Hungary) (100 µL per well) diluted 1:500 in acetate buffer (0.1M, pH 5.5) at 4°C overnight. Subsequently, microtitre plates were washed four times with a solution containing 22 g/L NaCl and 0.05% Tween-20 (PBS/N/T). A blocking step was performed with 200 µL per well blocking solution (1% BSA in PBS/N/T) for 30 min at room temperature, and washing was performed as above.

100µL of extracts of jejunum contents (jejunum contents diluted 1:10 in blocking solution) were added in duplicate onto the plate, incubated at 37°C for one hour under agitation and washed as above. After one hour, 100 µL of biotin conjugated IgG obtained from rabbits immunized with a recombinant beta2 toxin (CEVA-Phylaxia) and diluted 1:500 in blocking solution was added to each well and incubated at 37°C for 1 hour with agitation. After washing, 100 µL extravidin-peroxidase (Sigma), diluted 1:6000 in blocking solution was added and plates were incubated at 37°C for 30 min. Subsequently, 100 µL of TMB (Kem-Entec, ready to use) was added per well. Plates were covered to block out light and incubated at room temperature without agitation. The enzymatic reaction was stopped after 4 to 10 min by adding 50 µL of 0.5M H2SO4 per well. The result of the test was read on an automatic plate reader by measuring the Optical Density (OD) at 450 nm.

Serial two-fold dilutions from 1/2000 to 1/6400 of beta2 antigen standard (F05/16; kindly provided by B. Kadra, CEVA-Phylaxia, Hungary) diluted in PBS/N/T/1%BSA were used as positive control; no antigen was added in the wells used for negative control.

The cut-off of the assay was set at the mean OD of negative control replicates plus three standard deviations. The performance of the ELISA-test was not validated on toxin-negative intestinal contents or tested on spiked samples.

3.9.4 Methods used to detect viruses

Rotavirus

Contents of jejunum from all Case and Control piglets were examined for the presence of rotavirus A by ELISA (ProSpectT® Rotavirus) as described by the manufacturer.

Coronavirus

Case and Control piglets were tested by a conventional pan-corona RT-PCR assay designed to detect a wide range of coronaviruses (primer set: Cor-FW (5´-ACWCARHTVAAYYTNAARTAYGC-3´) and Cor-RV (5´-TCRCAYTTDG GRTA RTCCCA-3´, where Y=C/T, W=A/T, V=A/C/G, R=A/G, H=A/T/C, N=A/C/T/G (Eurogentec, Seraing, Belgium) (Vijgen et al., 2008). By a mistake (not recognised at submission of Manuscript 1), ten piglets were not tested (1 case piglet from Herd 1 and 9 piglets (5 cases and 4 controls) from Herd 4.

Prior to testing, RNA was extracted from ileum with contents. Samples were homogenized in 300 µl chilled 1-Thioglycerol Homogenization Solution (Promega) on a TissueLyserII (QIAGEN) at 20 Hz for 2 min, vortexed and heated at 70 °C for 2 min and then stored on ice. 300 µl of lysisbuffer was mixed into to the sample and 10 µl DNase added afterwards. RNA was extracted from all of the homogenate on a Maxwell® automated purification robot with the Maxwell® 16 LEV simplyRNA Tissue Kit (Promega) according to instructions from the supplier and eluted in 70 µl nucleic free water.

3.9.5 Methods used to detect parasites

Any presence of endogenous stages of *Cryptosporidium spp*, *Giardia spp*, *Cystoisospora suis* and *Strongyloides ransomi* was evaluated histologically using standard morphologic criteria.

3.10 Epidemiological methods for describing the syndrome

Epidemiological studies were based on clinical registrations from the first five days of life (the period when piglets within all herds were examined) and weight data from day one and day ten. Weight data at weaning turned out to be less useful since many (n=65) piglets were excluded from the study in the period between day 10 and weaning (mainly due to the decision to exclude small litters (with < 5 piglets left)).

The approach for describing the syndrome was to;

- Describe the sows and piglets in the study in terms of general characteristics and clinical findings
- Describe the course of diarrhoea within the herds in terms of prevalence, timing and duration as well as the tendency to cluster within litters
- Describe the timing of deaths and necropsy findings within the herds
- Evaluate the effects of diarrhoea on average daily gain (ADG) and mortality taking relevant co-variables into account and
- Evaluate sow- and piglet-level risk factors associated with diarrhoea taking relevant co-variables into account

During the preliminary screening of data it became clear that diarrhoea on the day of birth was common, and often seen in piglets not having diarrhoea later in the study period. Since it was not known if having liquid faeces on the day of birth was a normal phenomenon, not associated with disease, a four-level categorization of diarrhoeal status (see Table 3.4) was used in the studies on effect.

Data was clustered in a hierarchical structure (with the three levels; herds, litters and piglets), and mixed models were therefore used in the statistical evaluations. Models were fit using the lme4 package (Bates et al., 2013) in R (CoreTeam, 2013). In the models, litter was inserted as random effect. Herd was inserted as a fixed effect, since the specific effects of separate herds were of interest. In the generalized linear mixed (= logistic) models used in the studies on mortality and risk factors, population averaged Odds Ratios (OR_{PA}) were calculated since the aim was to draw general rather than litter-specific conclusions.

3.10.1 Effects of diarrhoea on ADG and mortality

Table 3.4 presents all explanatory variables inserted in the full models on ADG and mortality.

Table 3.4. Explanatory variables addressed in mixed models on ADG and mortality.

Explanatory variable	Level	Categorization
Herd	1,2,3,4	
Primary		
Diarrhoea	None	The piglet was not diarrhoeic at any day of the study
	1 day (during day 1)	The piglet was diarrhoeic for one day - at the day of birth
	1 day (during day 2-5)	The piglet was diarrhoeic for one day during the second to fifth day of life.
	>1 day	The piglet was diarrhoeic for more than one day during the five day study-period
Secondary		
Litter level		
Parity	Young	1st parity
	Mature	2nd – 7th parity
Diarrhoeal status of litter (DSL)	Severely affected	50% or more of the piglets in the litter were diarrhoeic for one or more days (the day of birth did not count)
	Mildly affected	Less than 50% of the piglets of the litter were diarrhoeic for one or more days (the day of birth did not count)
Piglet level		
Gender	Male	
	Female	
Birth weight	Continuous scale	
Skin abrasions	Yes	Skin abrasions on head or fore-knees at any point during the study period
	No	No skin abrasions on head or fore-knees

3.10.2 Risk factors associated with NNPDS

In this study, NNPDS was defined as diarrhoea at any point during the second to fifth day of life. The study focused on evaluating if factors present on the first day of life (including the presence of liquid faeces) were risk factors for development of NNPDS. Table 3.5 presents the risk factors evaluated in the study.

Table 3.5. Risk-factors for development of NNPDS that were addressed in mixed models.

Risk-factors	Level	Categorization
Herd	1,2,3,4	
Sow-related factors		
Parity	Young	1st parity
	Mature	2nd-7th parity
Litter size[1]	Large	Gilts: >15 piglets, Sows: >18 piglets
	Small	Gilts: <16 piglets, Sows: < 19 piglets
Stillborn	Many	>1 piglet
	Few	0-1 piglets
Clinical disease	Yes	Mastitis and/or temp>39.5°C and/or leg problems and/ or vulva discharge[2]
	No	None of the above
Piglet-related factors		
Gender	Male	
	Female	
Birth weight	Continuous scale	
Faecal consistency	Liquid	Watery or liquid consistency of rectal contents
	Normal	Creamy, firm or solid consistency of rectal contents and if no faeces on swab
Flanks	Hollow	Area behind ribs turned inwards
	Normal	Area behind ribs followed the line of the ribs
Hair coat	Rough	Hair coat appeared dull
	Normal	Hair coat did not appear dull

[1]: Litters above mean size in the parity group were considered large. [2]: Mastitis: One or more udder section hard, red or sore when palpated. Leg problems: The sow was unwilling to bear weight on all legs or sore at palpation. Vulva discharge: An excess of unclear or foul-smelling discharge.

4 Results obtained in the project

4.1 Herds

Table 4.1 gives a summary description of production data, health status and normal herd routines in the four herds of the study.

Table 4.1. Data on the four study-herds.

	Herd 1	Herd 2	Herd 3	Herd 4
Study period	January 2011	March 2011	May 2011	July 2011
Herd size	900 sows	1250 sows	700 sows	950 sows
SPF-status	None	None	SPF + Ap12	SPF
Liveborn/ litter[1]	15,7	14,3	14,0	16,2
Dead until weaning[1]	16,5%	16,4%	19,6%	15,3%
first parity litters[1]	20%	22%	21%	23%
Recruitment of gilts	Purchase	Own production	Purchase	Own production
Insemination by	Purchased semen	Own boars	Own boars	Purchased semen
Sowfeed[2]	Liquid, residue free/ Home made	Liquid, residue free/ Home made	Liquid, residue free/ Home made	Liquid/ Factory made
Vaccine[3]	Suiseng	Porcilis® coli 6C	Toxicol® vet	Porcilis® coli 6C
Routine treatment of piglets[4]	None	None	Amoxicillin at birth	Amoxicillin at castration
Antibiotic used for treatment of diarrhoea[5]	lincomycin/spectinomycin	sulfadiazine/ trimethoprim	sulfadiazine/ trimethoprim	colistin
Routine treatment of sows[6]	None	None	Oxytocin after farrowing	Oxytocin after farrowing
Antibiotic used for treatment of MMA[7]	sulfadiazine/ trimethoprim	sulfadiazine/ trimethoprim	sulfadiazine/ trimethoprim	sulfadiazine/ trimethoprim

[1]: Average calculated from herd-registrations made in a 3 month period prior to investigation. [2]: Feed type used in farrowing period. [3]: Sow-vaccine against *E.coli* and *C. perfringens type C* used during the study-period [4]: Standard antibiotic treatments used in the herds during the first week of life. These treatments were not used during the study. [5]: During the study, antibiotic treatment of diarrhoea was allowed after day 3 of life. [6]: Any standard medication used on the day of parturition. These treatments were continued during the study. [7]: During the whole study-period, medication of sows was allowed if considered necessary by the herd-manager.

4.2 Data used in the studies

A total of 989 piglets from 86 litters in the four herds were included at birth. Only piglets with a weight of minimum 800 g at birth were included.

At the age between three and seven days, a total of 110 piglets were removed from the herds to be euthanized for diagnostic purposes in the Case-Control study. Fig 4.1 shows the origin of data for the studies on weight gain and mortality. Since 32 piglets died prior to day five, 842 piglets were left for clinical examination on this day.

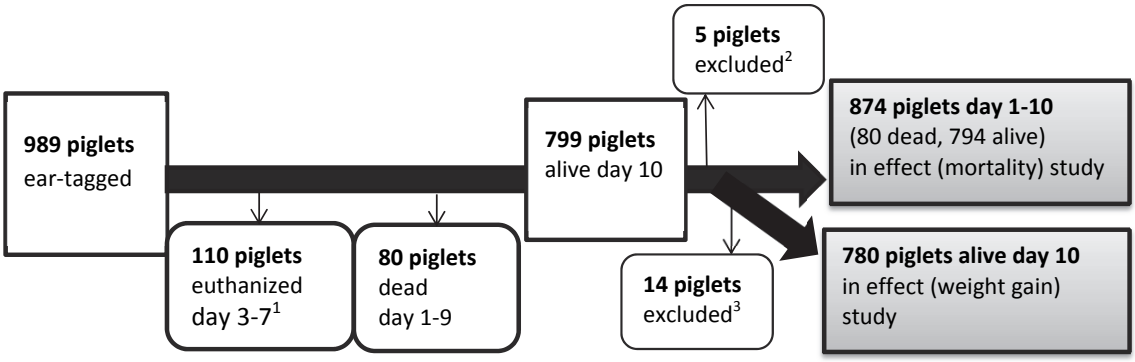


Fig. 4.1 Origin of data for studies on mortality and weight gain. Grey boxes show the number of piglets included in the separate studies.

[1]: These piglets were euthanized for diagnostic purposes in the Case- Control study. [2]: Excluded due to hermaphroditism or missing data. [3]: Excluded because their litters of origin were reduced in size (< 5 piglets).

The study on risk-factors associated with NNPDS included 941 piglets. A total of 47 piglets that died or were euthanized prior to day five with no history of diarrhoea and one hermaphroditic piglet were excluded from this data-set.

4.3 Case-Control investigations on euthanized pigs

In total, 51 diarrhoeic Case piglets (13, 11, 14 and 13 from Herds 1, 2, 3 and 4) and 50 non-diarrhoeic Control piglets (13, 12, 13 and 12 from Herds 1, 2, 3 and 4) at three to seven days of age were included in these investigations (nine piglets were mistakingly selected in the herds). Independent on the time-point of selection (early vs. late in the herd course of disease) the vast majority (80%) of the Case piglets had been diarrhoeic for two or three days prior to euthanasia. Out of 51 Case-piglets 31 were unmedicated, whereas 20 piglets (aged 5-7 days) were treated with antibiotics prior to euthanasia. All Control-piglets (n=50) were unmedicated.

Approximately half of the Case piglets from all herds did not have liquid contents in colon at the point of necropsy (one to six hours after being recognised with diarrhoea in the herds). These piglets may have been in remission of symptoms at selection.

4.3.1 Gross and histological findings

The most prominent necropsy finding in diarrhoeic piglets was intestinal flaccidity involving both the small and large intestine. Seen as a whole (calculated across herds), diarrhoeic piglets differed significantly ($P<0.05$ in Fisher's exact test) from non-diarrhoeic piglets with respect to body condition, hydration status, flaccidity of intestines and consistency of intestinal contents. Extra-intestinal gross lesions were infrequent in both groups of piglets. In case piglets, tear secretion (n=1), swollen kidneys (n=1), synovitis (n=1) and an icteric liver (n=5) were registered, whereas in control piglets synovitis (n=1) and an icteric liver (n=1) were the only extra-intestinal lesions.

Villous atrophy with crypt hyperplasia was the most frequent histological lesion. When calculated across herds, villous atrophy and epithelial lesions (in both the small and large intestine) were statistically associated ($P< 0.05$ in Fisher's exact test) with diarrhoea.

Necropsy and histological findings in piglets varied between herds. Important differences included;

- In Herd 2, neither Case nor Control piglets were dehydrated at necropsy. In the other herds, dehydration was evident in up to 57% of Case piglets.
- Intestinal flaccidity in Case piglets was less prevalent in Herd 4 than in the other herds, whereas flaccidity of intestines in Control piglets was relatively prevalent in this herd.
- In Herd 4, watery small intestinal contents were mostly seen in Control piglets. In Herd 3, this finding was equally prevalent in Case and Control piglets.
- Dullness of intestinal mucosa was primarily seen in piglets from Herd 1. Within this herd, 31% (n=4) of Case piglets and 15% (n=2) of Control piglets had this lesion. Within Herd 3 and Herd 4 this finding was only seen in single piglets (1 Control piglet in each herd), and it was not seen in piglets from Herd 2.
- In Herd 4, villous atrophy was equally prevalent in Case and Control piglets (approximately 35% of all piglets within Herd 4 had this lesion). In the other herds, villous atrophy was almost exclusively seen in Case piglets (around 70% of Case piglets vs. 8% of Control piglets within Herds 1-3 had this lesion).
- Epithelial lesions were primarily seen in Case piglets from Herd 2 and Herd 3. In Herd 1 and Herd 4, this finding was rare and not associated with diarrhoea.
- Neutrophil infiltration was observed in twice as many Case piglets than Control piglets in Herd 2, whereas in the other herds, the prevalence was similar in the two groups.
- Mucosal necrosis was seen in a few piglets from Herd 1 (n=1) and Herd 4 (n=2), but not in piglets from Herd 2 and 3. Both *Cp* and *F. necroforum* were detected by FISH within the necrotic lesion in one piglet from Herd 4, whereas *F. necroforum* was the only bacterium detected within the necrotic lesions in the piglet from Herd 1. In the second piglet from Herd 4, no bacteria were detected within necrotic lesions.

4.3.2 Detection of infectious agents

Bacteria – detected by culture and FISH

The prevalence of bacterial agents in 51 Case and 50 Control piglets as detected by culture and FISH is presented in Table 4.2. The FISH results are displayed as number of intestines with adherent bacteria (*E. coli* and *Enterococcus spp*) or bacteria situated in close proximity to the mucosal surface (*C. perfringens* and *C. difficile*). Antibiotic treatment of the older Case piglets may have induced a bias in the study. However, when comparing the detection rate of *E. coli* and *C. perfringens* in young (unmedicated (n=31)) and old (medicated (n=20)) Case piglets no obvious trend towards negative bacteriological results in the older piglets was recognized.

Table 4.2. Bacteria detected by culture and FISH in Case and Control piglets.

Bacterial agent	Case-piglets n=51		Control-piglets n=50	
Bacteria detected by culture	n	%	n	%
Haemolytic *E. coli* non-typable	3	6	0	0
Non-haemolytic *E. coli* non-typable	24	47	24	48
Non-haemolytic *E. coli* O-rough	2	4	1	2
Non-haemolytic *E. coli* O8	2	4	3	6
Non-haemolytic *E. coli* O157[1]	4	8	0	0
C. perfringens type A	18	35	35	70
C. perfringens type C[2]	3	6	1	2
C. difficile[3]	0	0	2	4
Bacteria detected by FISH				
E. coli	17	33	7	14
Enterococcus spp.	14	27	1	2
C. perfringens	10	20	15	30
C. difficile	0	0	0	0

[1]: All four isolates of non-haemolytic *E. coli* O157 were detected in Herd 3.

[2]: *C. perfringens* type C were detected in Herd 1 and Herd 4.

[3]: Both isolates of *C. difficile* were detected in Herd 1.

The tendency of Control piglets to have a higher detection rate of *Cp*A than Case piglets was the same within Herd 1, 2 and 3. In these herds, 3 (23%), 2 (18%) and 4 (29%) of Case piglets were positive, compared to 8 (62%), 6 (50%) and 12 (92%) of Control pigs. In Herd 4, the prevalence was the same in the two groups (9 Cases (69%) and 9 Controls (75%) were positive).

C. perfringens were detected by FISH in a total of 73% of Case piglets vs. 78% of control piglets (data not shown). In both Case and Control piglets, bacteria were most often seen within the intestinal contents. However, in approximately 25% of piglets, irrespective of diarrhoeal status, bacteria were detected in close proximity to the mucosal surface.

C. difficile were detected in small amounts in 60-70% of piglets in both groups (data not shown), however, were never seen in close proximity to the mucosal surface.

E. coli and *Enterococcus spp* were rarely detected in close proximity to the epithelium. However, within Herd 2, adherent *E. coli* were detected in 10 Case piglets (91%) vs. no Control piglets. In the same herd, adherent *Enterococcus spp* were detected in 8 Case piglets (73%) vs. no Control piglets. Cultural results in the 10 piglets with adherent *E.coli* detected by FISH interestingly showed that one of these piglets was negative by culture (a few non-haemolytic *E. coli* were detected in both intestinal segments). In the remaining nine piglets moderate to massive growth of non-haemolytic *E. coli* was detected (non-typable (n=8) and O-rough (n=1)). All these isolates were EAST-1 positive, but only three of them contained fimbrial genes, and no non-fimbrial adhesins were detected.

Apart from bacteria subsequently identified as either *E. coli*, *C. perfringens*, *C. difficile*, *enteroccus spp* or occasionally *F. necroforum* (see previous section), the universal probe (*Domain bacteria*) detected a large amount of bacteria within the luminal contents of all piglets (for a detailed description see Jonach et al 2014). However, no unidentified bacterial species were detected to adhere to the intestinal mucosa.

Virulence genes in E. coli isolates

A total of 63 isolates of *E. coli* were tested by PCR for the presence of virulence genes. However, the four non-haemolytic O157 isolates (which did not carry any fimbrial, STa, STb, LT or VT2e genes) escaped the detection of AIDA-1, intimin, Paa and EAST-1. Therefore, the results summarized in Table 4.3 refer to a total of 59 isolates.

Table 4.3. Results of virulence gene detection in *E. coli* isolates from 31 Case piglets and 28 Control piglets.

	n	None detected	Adhesin[1] alone	ST[2]/LT[3]/VT2e[4] no adhesin	EAST-1[5] with or without adhesin	Fimbriae[6]+ VT2e
Case-isolates	31	7	5	4	15	0
Control-isolates	28	13	3	4	5	3

[1]: Adhesin refers to any of the following: F4, F5, F6, F18, F41, AIDA-1 or intimin. [2]: Heat-Stable toxin a or b. [3]: Heat-labile toxin. [4]: Verotoxin 2e. [5]: Enteroaggregative *E. coli* Heat-Stable Enterotoxin 1. [6]: The three isolates carried F4, F5 and F18 fimbrial genes, respectively.

In 23% of Case-isolates and 46% of Control-isolates no virulence genes were detected. Paa was not detected in any isolate, and the prevalence of other adhesin-genes (F4, F5, F6, F18, F41, AIDA-1 and intimin) was low. In total, 9 of 31 (26%) Case-isolates and 8 of 28 (29%) of Control-isolates carried one or more adhesin genes.

EAST-1 was the most prevalent toxin-gene detected. In total, 15 (48%) of Case-isolates and 5 (18%) of Control-isolates were EAST-1 positive. In many (35%) of the EAST-1 positive isolates, however, no adhesin- gene was detected. EAST-1 positive isolates were primarily detected in piglets from Herd 2. Within this herd, 10 of 10 Case-isolates and 1 of 6 Control-isolates were EAST-1 positive.

Detection of beta2-toxin

A total of 95 intestines (45 from Case piglets and 50 from Control piglets) were tested for beta2-toxin. Altogether, 58% of Case intestines vs. 46% of Control intestines were detected toxin positive. Table 4.4 presents results within separate herds.

Table 4.4. Detection of beta2-toxin in jejunum contents in Case and Control piglets.

	Herd 1		**Herd 2**		**Herd 3**		**Herd 4**		**In total**	
Piglet status	Case	Control	Case	Control	Case	Control	Case	Control	Case	Control
Toxin positive	6 (50%)	7 (54%)	5 (56%)	5 (42%)	6 (55%)	6 (46%)	9 (69%)	5 (42%)	26 (58%)	23 (46%)

No association between the detection of beta2-toxin and diarrhoea was detected (Fisher's exact test, α=0.05) across or within herds. Interestingly, beta2-toxin was also detected in intestines with no detection of *Cp* by culture. A total of 20 intestines with negative bacteriological culture of *Cp* were toxin positive.

Rotavirus and coronavirus

A single piglet (1%) was positive for rotavirus A in the ELISA test. All piglets were negative in the pan-corona conventional PCR.

Parasites

Endogenous stages of *Cryptosporidium spp*, *Giardia spp*, *Strongyloides ransomi* and *Cystoisospora suis* were not detected by histological examination of intestines in any piglet.

4.3.3 A piglet-level case-definition

The Case- Control study suggested the following preliminary case-definition on NNPDS; Non-haemorrhagic diarrhoea during the first week of life, with no association to known infectious agents and characterized by a milk-filled stomach and flaccid intestines at necropsy.

4.4 Epidemiological results

4.4.1 Sows

Body condition scores of 1, 2, 3 and 4 were assigned to 0%, 18%, 79% and 1% of animals, respectively. No important differences between herds were observed.

Performance of the sows within the four herds with respect to litter size and number of stillborn is presented in Table 4.5.

Table 4.5. Parity-distribution, litter sizes and number of stillborn of the sows included in the study.

Herd	1	2	3	4	Total
1st parity/ Mature sows	5/17	10/11	5/16	9/13	29/57
Litter size[1] Mean (sd)	18.6 (2.6)	16.2 (2.8)	17.3 (2.4)	18.3 (3.6)	17.6 (3)
Litter size 1st parity[2]	15.6 (1.1)[a]	14.4 (2)[a]	16.2 (2.4)[a]	15.8 (2)[a]	15.3 (2)
Litter size older sows[2]	19.5 (2.2)[a]	17.9 (2.3)[ab]	17.6 (2.3)[b]	20.1 (3.4)[a]	18.8 (2.7)
Stillborn Mean (sd)	2 (1.6)	1 (1.14)	1.7 (1.6)	1.3 (1.3)	1.5 (1.5)

[1]: Refers to the total litter size, including stillborn piglets. [2]: Different letters within rows indicate significant (P<0.05) difference in t-tests (Welch method).

Overall, clinical disease in sows on the day of parturition was rare. However, more of the sows in Herd 1 than in the other herds were febrile or had leg problems. In Herd 3, a total of 4 sows (19%) were registered with mastitis. Table 4.6 presents a summary of clinical findings in sows from the four herds.

Table 4.6. Clinical findings in sows on the day of parturition.

Herd	1	2	3	4	Total
Number of sows	22	21	21	22	86
Fever (≥39.5°C)	27%	14%	10%	9%	15%
Leg problems	23%	5%	0%	9%	9%
Mastitis	0%	0%	19%	5%	6%
Vulva discharge	0%	10%	0%	0%	2%

4.4.2 Piglet characteristics and general piglet health

Summary characteristics of piglets from the four herds are presented in Table 4.7.

Table 4.7. Data on piglets in the study.

Herd	**1**	**2**	**3**	**4**	**Total**
n	227	245	216	253	941
Males/ Females	124/103	125/120	112/104	129/124	490/451
Born by first parity sows	54	117	53	104	328
Birth weight, mean (sd)[1]	1.36 (0.3)[a]	1.26 (0.2)[b]	1.34 (0.3)[ab]	1.33 (0.2)[a]	1.31 (0.2)
Born by mature sows	173	128	163	149	613
Birth weight, mean (sd)[1]	1.44 (0.3)[a]	1.42 (0.3)[a]	1.47 (0.3)[a]	1.42 (0.3)[a]	1.44 (0.3)

[1]: Different letters within rows indicate significant differences in t-tests (Welch method).

Each of the symptoms; swelling of navels, hoof lesions, swelling of vulvae, sneezing and bite wounds (from sow) were registered in a total of 1-3 piglets within herds. Swelling/soreness at palpation of joints was registered in 3-14 piglets (1-6%) within herds. Shaking syndrome was seen in 2 piglets (1%) from Herd 2 and 21 piglets (10%) from Herd 3. Wounds on fore-knees were seen in approximately 35% of piglets in all herds.

4.4.3 Appearance of the diarrhoea

The diarrhoea was yellow in the majority of cases (70-80% of cases on individual days of the 5-day study-period). A few cases of black (0-2% of cases), white (2-15% of cases), green (3-13% of cases) and colourless (0-2% of cases) faeces were also observed. No obvious relations between colour of faeces and diarrhoeic status were observed. Debris (resembling undigested milk) and/ or mucus were apparent in 0.5-7% of cases, and faeces were foamy in 0-7% of cases on the individual days. Non-diarrhoeic faeces were very seldom (<1%) recognized to contain any of these admixtures, perhaps mainly due to the fact that debris, mucous and foam are harder to recognize in faeces with normal consistency. Blood was not recognized within faeces at any point.

4.4.4 Prevalence and duration of diarrhoea

The daily prevalence of diarrhoea in piglets born by first parity sows and mature sows within each herd is presented in Fig 4.1.

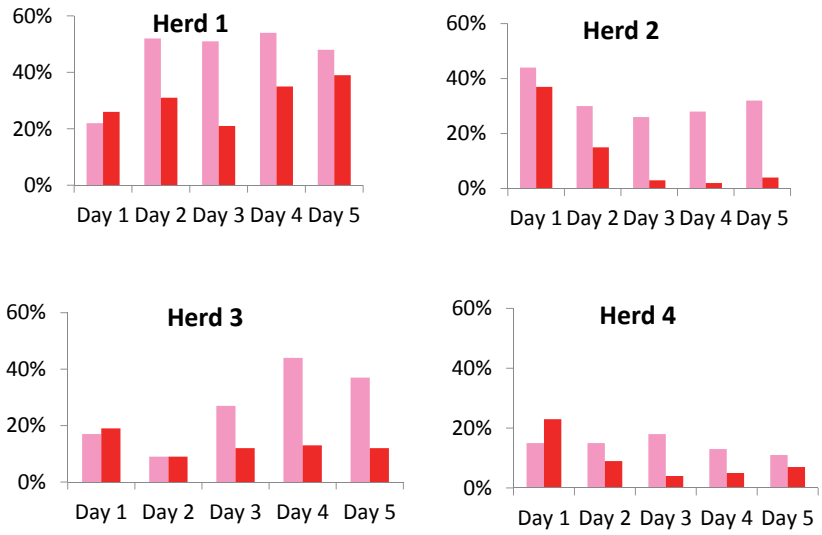


Fig 4.1. Daily prevalence of diarrhoea in 941piglets within the four herds of the study. Piglets with diarrhoea for more than one day appear more than once in the figure. Pink columns represent piglets born by first parity sows. Red columns represent piglets born by mature sows.

In many cases, when piglets were diarrhoeic for more days, symptoms were observed inconsistently. Thus, one day with normal faecal consistency observed in between days with diarrhoea was frequent. In the following, the term "duration" covers the number of days (during the first five days of life) that diarrhoea was observed in individual piglets. The duration of diarrhoea in piglets born by first parity sows and mature sows within the four herds is presented in Fig 4.2.

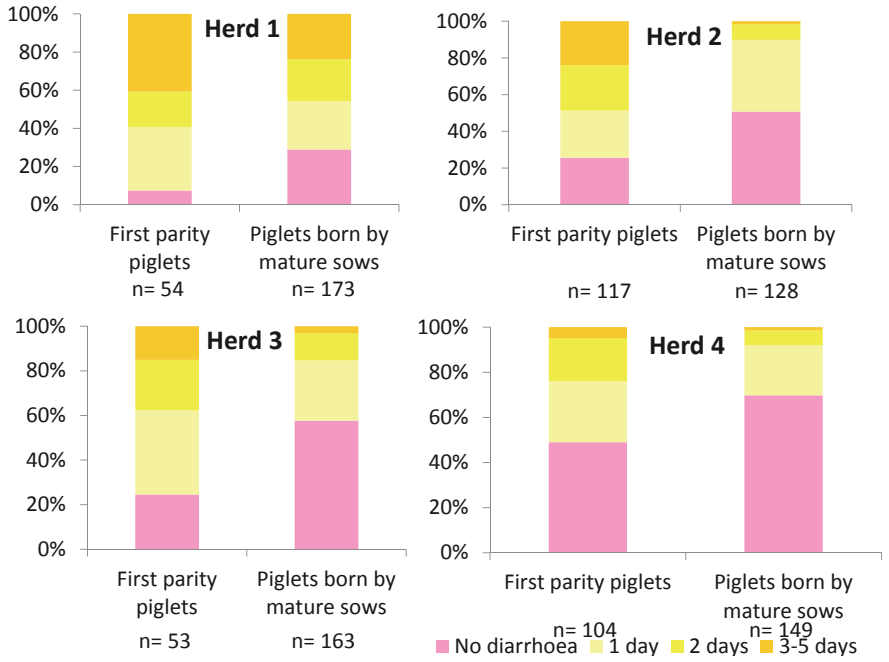


Fig 4.2. Duration (number of days during the first five days of life) of diarrhoea in 328 piglets born by first parity sows vs. 613 piglets born by mature sows in the four herds. The Y-axis shows the percentage of piglets.

The prevalence of diarrhoea was generally higher in piglets born by first parity sows and in piglets within Herd 1. Also, symptoms in individual piglets lasted longer in first parity piglets and in piglets within Herd 1. In the majority of cases, diarrhoea was observed for one or two days in total.

A total of 267 pigs (28%) in the study were diarrhoeic for one single day. Among these, a total of 111 pigs (42%) only had diarrhoea on the day of birth. These findings motivated the four-level categorisation of piglets' diarrhoeal status previously presented (see Table 3.4).

Not counting symptoms on day one (the basis for this approach follows), most piglets experienced the first symptoms on the second or third day of life. However, in Herd 3, symptoms started later than this in 50% of cases (data are shown in Manuscript 3).

4.4.5 Effects of diarrhoea on clinical appearance

Day five clinical registrations on parameters considered indicative for failure to thrive are presented in Table 4.8.

Table 4.8. Clinical signs of failure to thrive in 842 piglets alive on the fifth day of life.

	Days with diarrhoea			
Clinical finding[1]	**None** (n=377)	**1 day**		**>1 day** (n=204)
		During day 1 (n=111)	During day 2-5 (n=150)	
Hollow flanks	18%	10%	23%	38%
Protruding ribs	3%	2%	9%	22%
Dull hair coat	38%	27%	41%	50%
Dehydration	0%	0%	3%	15%

[1]: Definitions used: Hollow flanks: The area behind the ribs deviated inwards; Protruding ribs: Ribs and spine were visible; Dull hair coat: Hair coat appeared dull and longer than normal; Dehydration: sunken eye-balls and lack of skin-elasticity.

These findings indicated that the group of piglets with diarrhoea for >1 day were negatively affected on all registered clinical parameters. Individual piglets, however, did not constantly show obvious symptoms of failure to thrive.

4.4.6 Effects of diarrhoea on average daily gain (ADG)

The diarrhoea-associated variables (Diarrhoea and Diarrhoeal Status of Litter (DSL)) both had significant ($P=0.01$ and $P<0.001$) effects on ADG. Being part of a severely affected litter had a markedly larger effect (-38 g per day) than the individual status of the piglet (-9- -14 g per day, depending on the duration of diarrhoea). Piglets that were only diarrhoeic on the day of birth were not negatively affected on ADG compared to non-diarrhoeic ones. A very large influence on weight gain by litter of origin was observed (ICC=42%). In pairwise comparisons, ADG in non-diarrhoeic piglets was significantly different from ADG in piglets having diarrhoea for >1 day. Also, ADG in piglets only being diarrhoeic at the day of birth was significantly different from ADG in piglets having diarrhoea for >1 day.

ADG was not influenced by herd of origin, parity of sow, gender or presence of skin abrasions (other disease parameters were not statistically evaluated due to low prevalence and/or obvious herd associations).

4.4.7 Effects of diarrhoea on mortality

The overall mortalities in piglets studied within herds 1, 2, 3 and 4 were 21%, 7%, 6% and 4%, respectively. The mortalities within the four different diarrhoeal categories in each of the herds are presented in Table 4.9.

Table 4.9. Mortalities registered in each of the diarrhoeal categories within the four herds.

		Days with diarrhoea							
Herd	N total	None		1 day				>1 day	
				During day 1		During day 2-5			
		n	%[1]	n	%[1]	n	%[1]	n	%[1]
1	207	49	6	15	27	49	22	94	27
2	228	88	6	48	2	33	6	59	12
3	202	106	8	22	5	43	5	31	6
4	237	150	2	30	3	32	6	25	12
In total	874	393	5	115	6	157	11	209	18

[1]: % mortality in the group.

Within Herd 1, approximately 25% of the diarrhoeic piglets (independent of timing and duration of diarrhoea) died vs. 6% of the non-diarrhoeic ones. In the other herds, the mortalities were relatively low in both the diarrhoeic piglets (2-12%) and the non-diarrhoeic piglets (2-8%).

When herd of origin, litter of origin, parity of sow, birth weight and gender were taken into account, diarrhoea was not a significant risk factor for dying. Herd of origin was the most important factor associated with mortality and piglets born in Herd 1 were estimated to have an increased probability of dying of 11.8 compared to piglets from Herd 4. Descriptive data indicated that the surplus mortality within Herd 1 was caused by diarrhoea.

4.4.8 Categorisation of piglets for risk-factor assessment

From the descriptive results and from the results of the study on ADG it seemed reasonable to hypothesize that diarrhoea on the day of birth might be a normal phenomenon, unrelated to the investigated syndrome. This hypothesis, however, needed further evaluation in terms of assessing whether diarrhoea at birth was associated with diarrhoea on the subsequent days.

In the following text, the terms “liquid faecal consistency day one” and “NNPDS” are used to describe diarrhoea on the day of birth and diarrhoea at any point during day 2-5, respectively.

4.4.9 Prevalence of NNPDS within litters

The prevalence of NNPDS within litters in the separate herds is presented in Fig 4.3.

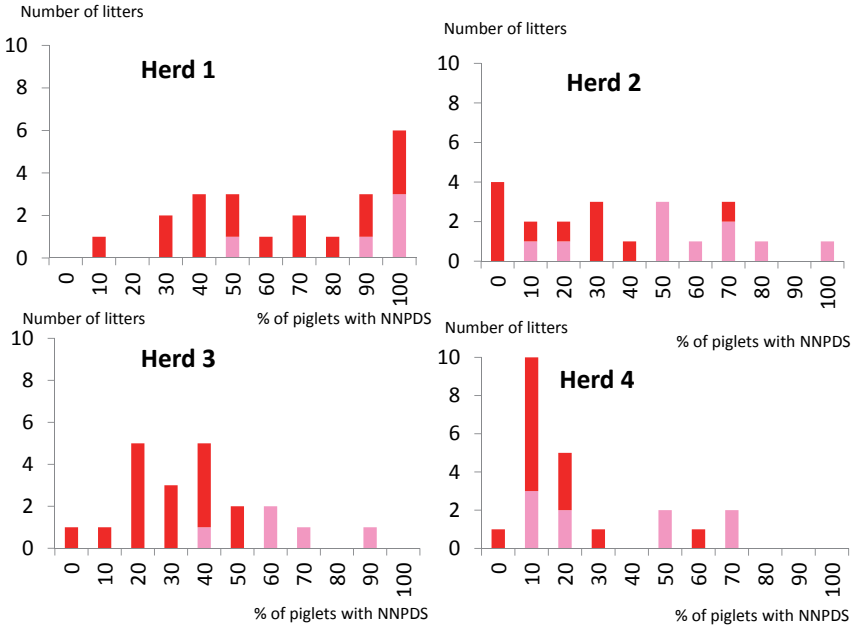


Fig. 4.3. Prevalence of NNPDS within 86 litters in the four herds. The x-axis shows the percentage of diarrhoeic piglets within a litter. The Y-axis shows the number of litters. First parity litters are presented in pink. Since each litter contained between 9 and 12 piglets, a 10% prevalence approximately equals one piglet.

All first parity litters in the study were affected by NNPDS. However, in Herd 2 and Herd 4, some of the first parity litters were very mildly affected (had 1-2 NNPDS affected piglets). In Herd 1 and Herd 3, 40-100% of piglets within first parity litters were affected by NNPDS. Only a few litters of mature sows (primarily within Herd 1), were severely affected. Herd 4 stood out as the least affected herd. A total of 11 litters (50%) within this herd were unaffected or only had a single piglet with NNPDS.

4.4.10 Risk factors

Herd of origin was the most important risk factor, with a 12.8 times increased risk of developing NNPDS in piglets within Herd 1 compared to piglets in Herd 4. Parity was also an important risk factor with an OR_{PA} of 4.1 in 1st parity piglets compared to piglets born by mature sows. Piglet-level effects were evaluated in Herd-specific models. These models showed a large variation in random litter effects between herds (ICC between 1% (herd 3) and 39% (herd1)). The odds of developing NNPDS were increased by 1.1 per 100 g decrease in birth weight. Liquid consistency of faeces day one increased the odds of developing NNPDS by

1.7. Effects of birth weight and faecal consistency day one were comparable in size between herds, however, significant within single herds only.

5 Manuscripts

Manuscript 1

Microbiological, pathological and histological findings in four Danish pig herds affected by a new neonatal diarrhoea syndrome.

Kongsted H., Jonach B, Haugegaard S., Angen Ø., Jorsal S.E., Kokotovic B., Larsen L.E., Jensen T.K. and Nielsen J.P. 2013. BMC Veterinary Research 9:206

Manuscript 2

The effect of Neonatal porcine Diarrhoea syndrome (NNPDS) on average daily gain and mortality in four Danish herds.

Kongsted H., Stege H., Toft N. and Nielsen J.P. 2014. BMC Veterinary Research 10:90

Manuscript 3

Risk factors and epidemiological characteristics of new neonatal porcine diarrhoea syndrome in four Danish herds.

Kongsted H., Toft N. and Nielsen J.P. 2014. BMC Veterinary Research 10:151

Manuscript 4

No evidence of a viral involvement in the new neonatal porcine diarrhoea syndrome in Danish pigs.

Larsen L.E., Hjulsager C.K., Boye M., Rasmussen S., Granberg F., Fischer T.K., Midgley S.E., Rasmussen L.D, Kongsted H., Angen Ø., Nielsen J.P. Under preparation for submission to Veterinary Microbiology.

Photo: Martin Dam Kristensen

5.1 Manuscript 1

Microbiological, pathological and histological findings in four Danish pig herds affected by a new neonatal diarrhoea syndrome.

Kongsted H.*, Jonach B, Haugegaard S., Angen Ø., Jorsal S.E., Kokotovic B., Larsen L.E., Jensen T.K. and Nielsen J.P.

*Corresponding author:

Hanne Kongsted, Danish Pig Research Centre

Phone: +4533394931/ +4524949736

Email: hko@lf.dk

BMC Veterinary Research

RESEARCH ARTICLE Open Access

Microbiological, pathological and histological findings in four Danish pig herds affected by a new neonatal diarrhoea syndrome

Hanne Kongsted[1,3*], Beata Jonach[2], Svend Haugegaard[1], Øystein Angen[2], Sven E Jorsal[2], Branko Kokotovic[2], Lars E Larsen[2], Tim K Jensen[2] and Jens P Nielsen[3]

Abstract

Background: Neonatal diarrhoea is a frequent clinical condition in commercial swine herds, previously regarded to be uncomplicated to treat. However, since 2008 it seems that a new neonatal diarrhoeic syndrome unresponsive to antibiotics and common management practices has emerged. Routine laboratory examinations have not detected any pathogen related to this syndrome. The primary purpose of this study was to evaluate if well-known enteric pathogens could be associated with outbreaks of neonatal diarrhoea, thus question the hypotheses of a new syndrome. Furthermore, we wanted to evaluate macroscopic and microscopic findings associated with these outbreaks and if possible propose a preliminary piglet-level case-definition on syndrome New Neonatal Porcine Diarrhoea syndrome (NNPDS).

Results: Four well-managed herds experiencing neonatal diarrhoea with no previously established laboratory conclusion and suspected to suffer from New Neonatal Porcine Diarrhoea Syndrome, were selected. Within these herds, 51 diarrhoeic and 50 non-diarrhoeic piglets at the age of three to seven days were necropsied and subjected to histological and microbiological examination. Faeces were non-haemorrhagic. Neither enterotoxigenic *E. coli*, *Clostridium perfringens* type A or C, *Clostridium difficile*, rotavirus, coronavirus, *Cryptosporidium spp*, *Giardia spp*, *Cystoisospora suis* nor *Strongyloides ransomi* were associated with diarrhoea in the investigated outbreaks. Macroscopically, the diarrhoeic piglets were characterized by filled stomachs and flaccid intestines without mucosal changes. The predominant histological lesions were villous atrophy in jejunum and ileum. Epithelial lesions in colon were seen in one third of the case piglets.

Conclusions: The results of the study supported the hypothesis that a new neonatal porcine diarrhoea was present in the investigated herds, since no known pathogen(s) or management factors could explain the diarrhoeal outbreaks. Based on the findings in the four herds the following case-definition of NNPDS was suggested: Non-haemorrhagic diarrhoea during the first week of life, without detection of known infectious pathogens, characterized by milk-filled stomachs and flaccid intestines at necropsy.

Background

Neonatal diarrhoea is a well-known clinical condition, present at varying prevalence in most commercial swine herds. However, since 2008 field experiences on an apparently new diarrhoeic syndrome unresponsive to antibiotics and common management practices have been reported (personal communications, S.E. Jorsal, National Veterinary Institute, Technical University of Denmark and B. Svensmark, Pig Research Centre, Danish Agriculture & Food Council, Denmark). The emergence of a new neonatal diarrhoeic syndrome (by some authors referred to as New Neonatal Porcine Diarrhoea (NNPD) has been suggested in different countries [1-4]. A common feature of the reported cases is that known enteric pathogens cannot be associated with the clinical outbreaks in routine laboratory submissions.

Routine laboratory testing protocols may vary from region to region. Disregarding local procedures, the following

* Correspondence: hko@lf.dk
[1]Pig Research Centre, Danish Agriculture & Food Council, Vinkelvej 13, Kjellerup 8620, Denmark
[3]HERD – Centre for Herd-oriented Education, Research and Development, Department of Large Animal Sciences, University of Copenhagen, Groennegaardsvej 2, Frederiksberg C 1870, Denmark
Full list of author information is available at the end of the article

agents are usually included in diagnostic protocols for neonatal diarrhoea: Enterotoxigenic *Escherichia coli* (ETEC), *Clostridium perfringens* type A (CPA), *Clostridium perfringens* type C (CPC), *Clostridium difficile* (CD) rotavirus group A (RV) and coronavirus [1,5]. Parasites, which may be relevant to consider in relation to neonatal diarrhoea are *Cryptosporidium spp*, *Giardia spp*, *Cystoisospora suis* and *Strongyloides ransomi* [6-8]. Systematic investigations of piglets from herds affected by the apparently new diarrhoeic syndrome are lacking.

The overall aim of this study was to investigate whether a detailed microbiological examination of a larger number of piglets from affected herds could link the presence of neonatal diarrhoea with known enteric pathogens. Such associations would challenge the hypothesis that a new disease syndrome has evolved. Another aim was to determine if diarrhoeic piglets from different herds had characteristic and consistent gross and microscopic lesions to support the elaboration of a joint case definition of NNPDS.

The article describes the prevalence of well-known enteric pathogens in age-matched diarrhoeic- and non-diarrhoeic piglets from four herds affected by neonatal diarrhoea with no previously established laboratory conclusion. Furthermore, results of gross pathology and histopathology are presented. Summarizing these findings, the article suggests a case-definition on NNPDS.

Results

Epidemiologic data on piglets

A total of 51 diarrhoeic (11–14 pr. herd) and 50 non-diarrhoeic piglets (12–13 pr. herd) at the age of three to seven days were included in the study. Clinically, the diarrhoeas were non-haemorrhagic. Eighty percent of diarrhoeic piglets had been diarrhoeic for either two or three days prior to euthanasia. Diarrhoea for four days was seen in 14% of the diarrhoeic piglets whereas only 6% had been diarrhoeic for five days.

Microbiology

Table 1 summarizes the microbiological findings in relation to diarrhoeic status. None of the microbiological agents was significantly more prevalent in diarrhoeic than in non-diarrhoeic piglets.

Non-haemolytic *E. coli* was the predominant finding in the aerobic culture from both diarrhoeic and non-diarrhoeic piglets, whereas haemolytic strains were found in only three piglets in total (all of them diarrhoeic). The main part of *E. coli* isolates were non-typeable. Sixty-three *E. coli* isolates were subjected to virulence gene determination by PCR. Fimbrial genes were detected in nine of 35 isolates from diarrhoeic piglets. The fimbrial distribution among isolates was; F4 (n=2), F5 (n=1), F6 (n=1), F18 (n=1), F41 (n=2), F5/F6 (n=1) and F5/F41 (n=1). In non-diarrhoeic piglets fimbrial genes were detected in seven of 28 isolates. The fimbrial distribution among these isolates was; F4 (n=1), F5 (n=1), F6 (n=1), F18 (n=2) and F41 (n=2). Table 2 gives an overview of toxin genes detected in fimbriated and non-fimbriated isolates from the two groups of piglets. Classic ETEC with simultaneous occurrence of both fimbrial and toxin genes were detected in only one diarrhoeic piglet.

Table 1 Microbiological findings in diarrhoeic and non-diarrhoeic piglets

Microbiological agent	Diarrhoeic n=51 (%)	Non-diarrhoeic n=50 (%)	*P-value*[1]
Haemolytic *E. coli* non-typable	6	0	0.1
Non-haemolytic *E. coli* non-typable	47	48	0.5
Non-haemolytic *E. coli* O-rough	4	2	0.5
Non-haemolytic *E. coli* serogroup O8	4	6	0.8
Non-haemolytic *E. coli* O157	8	0	0.06
C. perfringens type A	35	70	0.9
C. perfringens type C	6	2	0.3
C. difficile	0	4	0.5
rotavirus group A	2	0	0.5
coronavirus	0	0	1

[1]: Positive associations with diarrhoea tested by One-sided Fisher's exact test.

In the anaerobic culture, CPA was a very frequent finding. These bacteria were more prevalent in non-diarrhoeic than in diarrhoeic piglets (70% vs. 35%).

Necropsy

Necropsy findings are presented in Table 3. Very few extra-intestinal lesions were observed (not shown), and only one of these; a pale or icteric liver, was observed in more than one piglet (5 of the diarrhoeic vs. 1 of the non-diarrhoeic piglets).

As indicated in Table 3, six findings showed a statistically significant higher prevalence in diarrhoeic versus non-diarrhoeic piglets. Table 4 outlines the prevalence of these six findings in the two groups of piglets within each herd. Within all herds, a poor body condition, flaccidity of the small intestine, flaccidity of the large intestine and liquid large intestinal contents seemed positively associated with diarrhoea (though not statistically significant in all cases, see Table 4). Flaccidity of the large intestine was in most cases (25 of 27 cases) seen in conjunction with small intestinal flaccidity. Figure 1 shows a flaccid and Figure 2 shows a normal intestine.

Histopathology

In the small intestine, villous atrophy with crypt hyperplasia was the most frequently observed lesion. Figure 3

Table 2 Occurence of toxin genes within fimbriated and non-fimbriated *E. coli* isolates from diarrhoeic and non-diarrhoeic piglets

E. coli isolates	n	STa[1]	STb[1]	LT[2]	VT2e[3]	STa/LT	STb/VT2e	LT/VT2e
From diarrhoeic piglets								
Fimbriated	9	1	0	0	0	0	0	0
Non-fimbriated	26	1	2	2	5	0	0	0
From non-diarrhoeic piglets								
Fimbriated	7	0	0	0	3	0	0	0
Non-fimbriated	21	1	0	0	3	1	1	1

[1]: Heat-stable enterotoxins a and b. [2]: Heat-labile enterotoxin. [3]: Verotoxin 2e.

shows atrophic villi in ileum as compared to normal villi shown in Figure 4. Overall, an atrophic pattern was seen in the jejunal and/or ileal mucosa in 63% of diarrhoeic and 12% of non-diarrhoeic piglets. The severity of atrophy varied, with no obvious association with diarrhoeic status. In ileum, the villous atrophy was most pronounced over the Peyer's patches. Duodenal villi were not affected. A statistically significant association (P<0.001) between villous atrophy and flaccidity of the small intestine at necropsy was seen. In 76% of piglets having villous atrophy, small intestinal flaccidity had been recorded at necropsy.

Irrespective of diarrhoeic status approximately 30% of piglets had a slight to moderate local infiltration of neutrophils in the lamina propria. Occasionally, the lamina propria in the diarrhoeic piglets was congested and edematous.

Mild epithelial lesions were seen at the tip of the villi in 20% of the diarrhoeic and 6% of the non-diarrhoeic piglets and were usually associated with villous atrophy. Crypts of Lieberkühn epithelium were intact in both groups. Foci of mucosal necrosis were seen in the small intestines of 6% of the diarrhoeic piglets versus none of the non-diarrhoeic ones. In colon, mild epithelial lesions were seen in 33% of the diarrhoeic piglets and 11% of the non-diarrhoeic piglets. Occasionally, the colonic crypts in diarrhoeic piglets were irregular and elongated. Mucosal necrosis in the colon was seen in one diarrhoeic piglet, which also had necrotic changes in the small intestine.

No parasites were seen in the intestinal mucosa of any piglet.

Table 5 depicts histopathological findings in diarrhoeic and non-diarrhoeic piglets within the four herds and summarizes the overall prevalences. Both villous atrophy and large intestinal epithelial lesions showed an overall statistically significant positive association with diarrhoea, and seemed positively (or at least not negatively) associated with diarrhoea within all herds.

Table 3 Necropsy findings in diarrhoeic and non-diarrhoeic piglets

Necropsy findings	Diarrhoeic n=51 (%)	Non-diarrhoeic n=50 (%)	*P-value*[1]
General findings			
Poor body condition	**57**	**4**	**< 0.0001**
Dehydration	**29**	**2**	**< 0.0001**
Empty stomach	0	12	1
Small intestine			
Flaccidity	**73**	**20**	**< 0.0001**
Hyperaemia of serosa	6	0	0.2
Striping of serosa	2	0	1
Edema in mesentery	4	4	1
Enlargement of lymph nodes	18	16	0.9
Dullness/ necrosis of mucosa	8	8	1
Watery contents	**57**	**30**	**0.01**
Large intestine			
Flaccidity	**53**	**6**	**< 0.0001**
Edema in mesentery	39	20	0.06
Enlargement of lymphnodes	4	2	1
Liquid contents	**48**	**10**	**< 0.0001**

[1]: One-sided Fisher's exact test. Findings having a statistically significant positive association with diarrhoea are presented in bold.

Discussion

Non-enterotoxigenic (containing neither fimbrial nor toxin genes) *E. coli* was a frequent finding in both diarrhoeic and non-diarrhoeic piglets, whereas only one enterotoxigenic isolate was detected. Hence, ETEC did not seem to play any pathogenic role in relation to the investigated outbreaks of diarrhoea. Other studies have indicated that attaching and effacing *E. coli* (AEEC), carrying neither fimbrial nor toxin genes, are able to induce diarrhoea in newborn piglets [9] and to induce villous atrophy [10]. The prevalence of AEEC in the present study is currently being investigated.

CPC was cultured in four piglets of the study. Due to the low prevalence the significance of this bacterium in relation to the investigated outbreaks is probably minimal. The significance of CPA in relation to diarrhoea in

Table 4 Necropsy findings in diarrhoeic- (D) and non-diarrhoeic (ND) piglets in individual herds

Necropsy findings	Herd 1		Herd 2		Herd 3		Herd 4	
	D	ND	D	ND	D	ND	D	ND
	n=13	n=13	n=11	n=12	n=14	n=13	n=13	n=12
	(%)	(%)	(%)	(%)	(%)	(%)	(%)	(%)
General findings								
Poor body condition	77*	15	63*	0	57*	0	31*	0
Dehydration	31*	0	0	0	57*	8	23	0
Small intestine								
Flaccidity	69*	15	81*	25	86*	0	54	42
Watery contents	92*	23	64*	17	36	31	38	50
Large intestine								
Flaccidity	62*	8	55*	8	64*	0	31	8
Liquid contents	46*	8	45	17	50*	8	46*	8

* denotes significant statistical positive association with diarrhoea (one-sided Fisher's exact test (α=0.05)) within the individual herds.

neonatal piglets is controversial, since it has been concomitantly recognized as part of the normal intestinal flora and as a potential intestinal pathogen [11,12]. In this study we found a significantly higher prevalence of CPA in non-diarrhoeic vs. diarrhoeic piglets. Most likely, this finding merely reflects the intact intestinal flora within the non-diarrhoeic piglets. CD has been reported in cases of neonatal diarrhoea in piglets [5,12]. However, in this study, this bacterium was only detected in two piglets and the characteristic histopathological lesions previously reported to be associated with CD infections [13] were not seen. Accordingly, CD does not seem to be associated with the investigated outbreaks.

The scarcity of known pathogens in the outbreaks of the study supports the hypothesis that the investigated herds experienced diarrhoea of unknown aetiology. Therefore the outbreaks may be representative of the new syndrome NNPDS.

A poor body condition and dehydration were rather prevalent findings in diarrhoeic piglets in this study. However, unless very pronounced, these features are not characteristic for specific diarrhoeic syndromes, since they merely reflect the loss of nutrients and water associated

Figure 1 Flaccid intestine of 3 days old diarrhoeic piglet. The small intestine is thin-walled and flaccid throughout its length. The intestine appears to lack its normal peristaltic capacity, since no sections are contracted. Colon (in the right side of the picture) has liquid contents.

Figure 2 Normal intestine of 3 days old non-diarrhoeic piglet. The small intestinal wall has a normal thickness. Different parts of the intestine show different stages of peristalsis, reflecting normal peristaltic capacity.

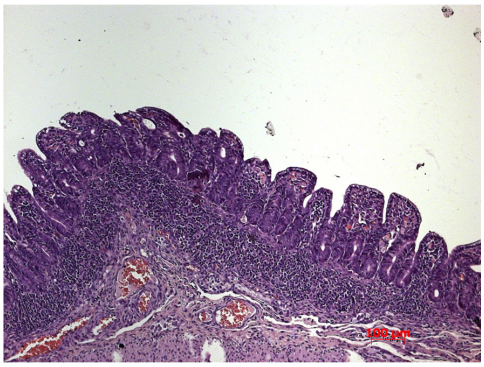


Figure 3 Severe villous atrophy in ileum of 5 days old diarrhoeic piglet.

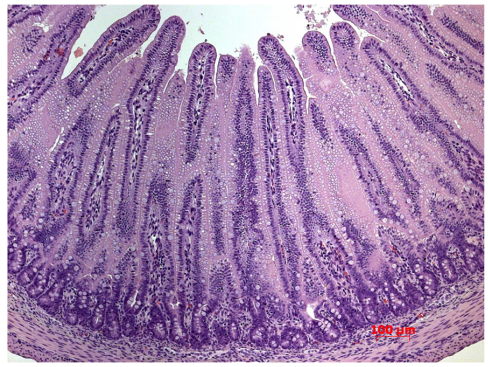


Figure 4 Normal intestinal villi in ileum of 5 days old non-diarrhoeic piglet.

with any diarrhoeic condition. Milk-filled stomachs, in contrast, seem to be a characteristic finding associated with this syndrome. Since neonatal diarrhoea is commonly associated with malabsorption caused by starvation, the filled stomachs seen in 100% of diarrhoeic piglets in this study are interesting findings which clearly differentiate this syndrome from outbreaks of neonatal diarrhoea related to starvation. However, since the vast majority (80%) of piglets in this study were diarrhoeic for two or three days only, we do not have information on the contents of stomachs at later stages of disease. Obviously, one would expect long lasting diarrhoea to keep piglets from suckling due to malaise, and therefore this criterion is probably only valid at early stages of disease.

Intestinal flaccidity was the most prominent and consistent gross lesion. Flaccidity of intestines is seen in different conditions, Postweaning Multisystemic Wasting Syndrome (PMWS) [14] and diet-induced malabsorption being the most obvious examples. Liquid contents in colon are expected in all diarrhoeic conditions and are therefore not considered diagnostic to any specific syndrome. Somewhat surprising, half of the diarrhoeic piglets did not have liquid content in colon at necropsy, which probably reflects that these piglets were in the recovery phase of disease. If so, this potentially implies a diagnostic problem due to less pronounced lesions and decreased excretion of infectious agents at this phase. However, since the clinical course of diarrhoea turned out to be very short (as evidenced by 80% of the selected piglets being diarrhoeic for only two or three days) it would not have been practically feasible to avoid selection of piglets in recovery.

The most consistent and predominant histological lesion observed in diarrhoeic piglets was villous atrophy (seen in 63% of diarrhoeic vs. 12% of non-diarrhoeal piglets). Villous atrophy is a very common finding in diarrhoeic conditions [15] and in this study, the atrophy was neither associated with infection by well-known pathogens nor malnutrition. The strong association between villous atrophy and grossly visible intestinal flaccidity indicates that decreased mucosal thickness is reflected grossly as a thin-walled, atonic intestine. Epithelial lesions in the large intestine also seemed to be consistently associated with diarrhoea in this study (seen in 33% of diarrhoeic vs. 11% of non-diarrhoeic piglets), but due to the low prevalence, these lesions do not seem to be relevant to include in a case definition.

Overall, the present study suffers from lack of comparable piglets from non-NNPDS-affected herds in order to correctly classify findings as typical or diagnostic of NNPDS. Moreover, the selection of study herds posed some difficulties since the selection was basically based on a high prevalence of diarrhoea and absence of agents (in five piglets). Obviously, potential misclassification of herds is an issue to consider – though hard to address or control at this stage of investigation.

To our knowledge, this is the first study investigating outbreaks of diarrhoea in herds suspected to suffer from NNPDS. The aetiology behind these outbreaks was either undetected pathogens or non-infectious factors. Practical experience indicates that eg. high levels of protein in sow feed can lead to diarrhoea in neonatal pigs. However, all of the investigated herds used restricted levels of protein in sow feed, and had previously tried minimizing protein content with no preventive effect. As previously underlined, the diarrhoea seemed unrelated to postnatal starvation, bot intrauterine events may have affected the normal development of intestinal absorptive capacity. Thus, the villous atrophy seen in the study may reflect prenatal under-development of villi.

The unspecific nature of intestinal lesions seen in this study underlines the complexity of intestinal pathology in neonatal pigs. Interestingly, even early studies from

Table 5 Main histological findings in 51 diarrhoeic (D) and 50 non-diarrhoeic (ND) piglets

Histological findings	Herd 1		Herd 2		Herd 3		Herd 4		In total	
	D	ND	D	ND	D	ND	D	ND	D	ND
	n=13	n=13	n=11	n=12	n=14	n=13	n=13	n=12	n=51	n=50
	(%)	(%)	(%)	(%)	(%)	(%)	(%)	(%)	(%)	(%)
Small intestine										
Villous atrophy	62*	8	82*	8	71*	0	38	33	**63**	**12**
Neutrophil infiltration	38	38	55	25	21	15	23	50	33	32
Epithelial lesions	15	15	36*	0	21	0	8	8	**20**	**6**
Mucosal necrosis	8	0	0	0	0	0	15	0	6	0
Large intestine[1]										
Epithelial lesions	17	15	55*	8	46	22	15	0	**33**	**11**
Mucosal necrosis	0	0	0	0	0	0	8	0	2	0

[1]: In 2 diarrhoeic and 4 non-diarrhoeic piglets samples from colon were missing or autolytic and therefore not included in the analysis.
*denotes statistically significant positive associations with diarrhoea within herds (one-sided Fisher's exact test (α=0,05)). Findings having a statistically significant association with diarrhoea across herds are presented in bold.

the seventies and eighties concluded that gross lesions seem similar and unspecific irrespective of the underlying aetiology in this age group of piglets [16,17]. Moreover, in the study from 1975 no pathogens were detected in as many as 32% of fatal neonatal gastroenteropathies. Thus, the existence of neonatal diarrhoa with unspecific lesions and without known pathogens is not a new phenomenon. It appears, however, that the clinical picture in the herds is new. At this point we do not know whether this clinical picture is a more severe manifestation of a syndrome already present but not recognized back in the seventies, or if we are experiencing a truly new syndrome.

From a practical point of view, obviously the most urgent issue is to recognize the aetiology behind the current problems. This study disclaims associations with established agents, but yet unestablished agents or shifts in intestinal bacteral population dynamics may play a role. Therefore, culture-independent methods like metagenomics and high-throughput qPCR may be rewarding in the future investigation.

Conclusions

This study suggests the existence of a yet unexplained diarrhoea syndrome related to the first week of life. The syndrome is not related to starvation or infection by enterotoxigenic *E. coli*, *Clostridium perfringens* type A or C, *Clostridium difficile*, rotavirus, coronavirus, *Cryptosporidium spp*, *Giardia spp*, *Cystoisospora suis* or *Strongyloides ransomi*. Characteristic postmortem findings are flaccid intestines without mucosal pathology or lymph node enlargement. Histologically, villous atrophy in jejunum and ileum are the most prominent findings and epithelial lesions in colon also seem to be associated with the syndrome. Apparently, the flaccidity of intestines seen at necropsy is a reflection of the reduced length of intestinal villi. For a preliminary piglet level case-definition we suggest the following; Non-haemorrhagic diarrhoea during the first week of life, with no detection of known infectious agents and characterized by a milk-filled stomach and flaccid intestines at necropsy. Since histopathological examination mainly revealed uncharacteristic lesions, this diagnostic approach does not seem to be rewarding at this stage of investigation.

Methods

Study design

A case–control study on 101 euthanized piglets selected from four Danish production herds was performed during 2011. A total of 989 piglets from these herds were clinically evaluated from the day of birth and 110 were euthanized at selected time points.

Inclusion of herds

Herds were recommended by veterinary practitioners and included in accordance with the following criteria: 1) Presence of diarrhoea responding poorly to antibiotics during the first week of life (at least 30% affected litters for a period of minimum 6 months), 2) Routine vaccination of sows against ETEC and CPC, 3) Failure of preventive management interventions, 4) PRRS negative farrowing unit as demonstrated in blood samples tested by ELISA/IPT or PCR and 5) Negative results of routine diagnostic examinations for ETEC, CPC and RV in five diarrhoeic piglets aged one to four days.

A total of four herds were selected. They all presented high standards of housing and management with all- in/all-out practice in farrowing units and appropriate cleaning between farrowing batches. Farrowing crates had partially slatted floors of plastic or iron bars with supplemental heat and cover provided for the piglets.

All four herds had been affected by neonatal diarrhoea for at least one year. Preventive interventions which had failed included minimizing protein-levels in sow feed, optimization of hygiene procedures, immunization by faecal backfeeding and vaccination against CPA and Porcine circovirus type 2 (PCV2). In all herds, many different antibiotics and different treatment strategies had been tried out unsuccessfully. All herds used Toltrazuril at day three to four of life to prevent coccidiosis. Castration of males and iron-injections were carried out at the same day. Descriptive data on the herds are presented in Table 6.

Inclusion of sows and piglets

In each herd, approximately 20 newly farrowed sows (half of a farrowing batch) with no clinical signs of disease prior to farrowing were selected. The selection procedure was designed to include all available first parity litters, since they were expected to exhibit the highest prevalence of diarrhoea. At the day of birth (day one), the included litters were standardized to 11 or 12 piglets by randomly selecting piglets weighing ≥ 800 g. Surplus piglets were removed during the first 16 hours after birth and no cross-fostering was made during the suckling period. Sows and piglets were clinically examined daily from day 1 until day five to seven and again on day ten. In the same period, rectal swabs were taken. Consistency of faeces was judged as fluid or normal from the appearance on the rectal swab.

Definition on cases and controls and selection procedure

In each herd, age-matched case and control piglets were selected for necropsy at two different time-points – early and late in the course of disease. These time-points were based on previous experience with the syndrome in each herd. In herds experiencing diarrhoea starting at the second day of life, piglets were necropsied at day three and five of life. If diarrhoea occurred from the third day of life, piglets were necropsied at day four and six of life and so on. In the 4 herds, clinical signs started at day two, three or four of life, resulting in necropsies being performed on piglets between three and seven days of age.

Selection criterion for diarrhoeic piglets was fluid consistency of faeces for at least two subsequent days, including the day of selection. Selection criterion for non-diarrhoeic piglets was normal consistency of faeces at all days prior to selection. Diarrhoeic piglets were selected from the litters having the highest prevalence of diarrhoea, whereas non-diarrhoeic piglets were selected from the litters exhibiting no or little diarrhoea. None of the piglets euthanized at the early stage (three to five days of age, depending on herd) had been treated by antibiotics. All diarrhoeic piglets euthanized at the late stage (five to seven days of age, depending on herd) had been medicated according to the individual herd routine.

Diagnostic procedures

Necropsy

Live piglets were transported to the laboratory and euthanized within six hours after selection. All organs were routinely examined for gross lesions. A poor body condition was recorded if protruding ribs and spine were observed. Dehydration was recorded if eye balls were deeply positioned in the skull and muscles appeared dry on the cut surface.

Histopathology

Histopathological examination was carried out on samples from duodenum, jejunum, ileum and spiral colon. Samples were fixed immediately after euthanasia in 10% neutral buffered formalin for at least 48 hours. The samples were then embedded in paraffin wax, cut at 3 μm, stained with haematoxylin and eosin (HE) and examined by light microscopy. The intestinal mucosa of each specimen was histopathologically evaluated.

Villous atrophy was recorded when shortening of villi accompanied by decreased height of enterocytes and increased cellularity of the lamina propria was seen in at least one region of the intestinal sample. Crypt hyperplasia was recorded when the intestinal crypts were

Table 6 Descriptive data of the 4 herds in the study

Herd data	Herd 1	Herd 2	Herd 3	Herd 4
Study period	January 2011	March 2011	May 2011	July 2011
Herd size (number of sows)	900	1250	700	950
SPF[1]-status	Not declared	Not declared	SPF+AP12	SPF
Piglets weaned/sow/year[2]	30.7	27.1	25.4	32.3
1st parity litters (%)[2]	20	22	21	23
Recruitment of gilts	Purchase	Own production	Purchase	Own production
Sow feed[3]	Wet/ Home made	Wet/ Home made	Wet/ Home made	Wet/ Factory made

[1]: Specific Pathogen Free - disease surveillance programme. Danish herds can participate in this programme which registers presence of certain infectious diseases, including PRRS, *M. hyopneumoniae, A. pleuropneumoniae, P. multocida* tox+ and *B. hyodysenteriae.* [2]: Average values calculated from herd-registrations made in a 3-month-period prior to investigation. [3]: Feed type used in farrowing period.

elongated with an increased number of mitotic figures. Disruptions in normal epithelial architecture with preserved integrity of the epithelium were recorded as mild epithelial lesions. Diffuse necrotic changes in the epithelium and lamina propria were recorded as mucosal necrosis.

The presence of parasites (*Cryptosporidium spp*, *Giardia spp*, *Cystoisospora suis* and *Strongyloides ransomi*) was examined using standard diagnostic criteria.

Bacteriology

Sections of jejunum and colon were aerobically cultured for *E. coli*. Parallel culturing on Drigalski (in house selective and indicative medium for coliforms) and blood agar plates (Columbia agar (Oxoid) supplemented with 5% calf blood) was performed. Plates were incubated for 24 hours at 37°C. Piglets were considered *E. coli* positive if any growth of haemolytic colonies or moderate/ massive growth of non-haemolytic colonies was seen in any section of intestine. Serogrouping of *E. coli* was performed - using one isolate per piglet - by agglutination with monovalent O-antisera (O8, O45, O64, O138, O139, O141, O149 and O157, Statens Serum Institut, Copenhagen, Denmark) [18] and real-time PCR was performed for detection of virulence factor genes F4, F5, F6, F18, F41, STa, STb, LT and VT2e [18]. If no agglutination with antisera was seen, the isolate was designated non-typeable. If agglutination occurred in all pools, the isolate was considered to be O-rough.

Culturing of CP was carried out using Columbia agar (Oxoid) supplemented with 5% calf blood and polymyxin incubated anaerobically for 24 hours at 37°C. Colonies were verified using Tryptose-Sulfite-Cycloserine agar (Oxoid). Piglets were considered culture positive if moderate/ massive growth was observed in any section of intestine. Typing of suspected samples was performed by PCR [19] on a pool of four isolates having characteristic colony morphology on Columbia agar (shiny, grey, double-haemolytic colonies). Culturing of CD was performed using Cycloserine Cefoxitin Fructose Agar, incubated anaerobically for 48 hours at 37°C. Piglets were considered positive if yellow colonies with a characteristic horse-stable odour were detected in any section of intestine.

Virology

Contents of jejunum were examined for rotavirus group A by an enzyme immunoassay (ProSpectT® Rotavirus) according to the manufacturer's instructions and for coronavirus by a pan-corona RT-PCR assay as previously described [20].

Statistics

Positive associations between diarrhoea and microbiological and pathological findings were evaluated using one-sided Fisher's exact tests (α=0.05). When considered relevant, associations between histopathology and necropsy were also evaluated by Fisher's exact tests (α=0.05). Since the piglets originated from four different herds, associations within herds were also assessed.

Ethical approval

The present study was not subject to ethical approval as Danish laws do not require ethical approval for studies not involving different treatment groups or blood testing. The study only involved procedures normally used for routine diagnostics.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

All authors contributed to the design of the study. Inclusion of herds, clinical examination in the herds and selection of piglets was done by HK. Necropsy was performed by SH and histological examinations were performed by BJ. Culturing of *Clostridium difficile* was done by BK and virulence gene determination of *E. coli* isolates by ØA. LEL did the pan-corona RT-PCR assays. HK conducted the statistical analysis. All authors participated in drafting the manuscript and proofreading of the final manuscript.

Acknowledgements

The study was supported by the Danish Ministry of Food, Agriculture and Fisheries. The authors wish to thank Birgitta Svensmark and technical personnel at the Laboratory of swine Diseases, Pig Research Centre and technical personnel at the National Veterinary Institute, Technical University of Denmark for their technical assistance.

Author details

[1]Pig Research Centre, Danish Agriculture & Food Council, Vinkelvej 13, Kjellerup 8620, Denmark. [2]National Veterinary Institute, Technical University of Denmark, Bülowsvej 27, Frederiksberg C 1870, Denmark. [3]HERD – Centre for Herd-oriented Education, Research and Development, Department of Large Animal Sciences, University of Copenhagen, Groennegaardsvej 2, Frederiksberg C 1870, Denmark.

Received: 4 March 2013 Accepted: 4 October 2013
Published: 12 October 2013

References

1. Svensmark S: **New neonatal diarrhoea syndrome in Denmark.** In *Proceedings of the 1st ESPHM*. Edited by Stege H, Kristensen CS. Copenhagen, Denmark: Faculty of Life Sciences, University of Copenhagen; 2009:27.
2. Gin T, Le Guennec J, Morvan H, Martineau G-P: **Clinical and laboratory investigations in 10 French pig herds dealing with enzootic neonatal diarrhoea.** In *Proceedings of the 21st IPVS Congres*. Edited by D'Allaire S, Friendship R. Vancouver, British Columbia, Canada; 2010:758.
3. Melin L, Wallgren P, Mattson S, Stampe M, Löfstedt M: **Neonatal diarrhoea in piglets from E. coli vaccinated sows in Sweden.** In *Proceedings of the 21st IPVS Congres*. Edited by D'Allaire S, Friendship R. Vancouver, British Columbia, Canada; 2010:290.
4. Wallgren P, Mattson S, Merza M: **New neonatal porcine diarrhoea. II. aspects on etiology.** In *Proceedings of the 22nd IPVS Congres*. Jeju, Korea; 2012:76.
5. Yaeger M, Funk N, Hoffman L: **A survey of agents associated with neonatal diarrhea in Iowa swine including Clostridium difficile and porcine reproductive and respiratory syndrome virus.** *J Vet Diagn Invest* 2002, **14:**281–287.
6. Vitovec J, Koudela B: **Pathogenesis of intestinal cryptosporidiosis in conventional and gnotobiotic piglets.** *Vet Parasitol* 1992, **43:**25–36.
7. Meyer C, Joachim A, Daugschies A: **Occurrence of Isospora suis in larger piglet production units and on specialized piglet rearing farms.** *Vet Parasitol* 1999, **82:**277–284.

8. Maddox-Hyttel C, Langkjaer RB, Enemark HL, Vigre H: ***Cryptosporidium*** **and** ***Giardia*** **in different age groups of Danish cattle and pigs - occurrence and management associated risk factors.** *Vet Parasitol* 2006, **141:**48–59.
9. Ngeleka M, Pritchard J, Appleyard G, Middleton DM, Fairbrother JM: **Isolation and association of Escherichia coli AIDA-I/STb, rather than EAST1 pathotype, with diarrhea in piglets and antibiotic sensitivity of isolates.** *J Vet Diagn Invest* 2003, **15:**242–252.
10. Helie P, Morin M, Jacques M, Fairbrother JM: **Experimental infection of newborn pigs with an attaching and effacing Escherichia coli O45:K"E65" strain.** *Infect Immun* 1991, **59:**814–821.
11. Songer JG, Uzal FA: **Clostridial enteric infections in pigs.** *J Vet Diagn Invest* 2005, **17:**528–536.
12. Lippke RT, Borowski SM, Marques SMT, Paesi SO, Almeida LL, Moreno AM, Corbellini LG, Barcellos DESN: **Matched case–control study evaluating the frequency of the main agents associated with neonatal diarrhea in piglets.** *Pesquisa Veterinaria Brasileira* 2011, **31:**505–510.
13. Keel MK, Songer JG: **The Comparative Pathology of Clostridium difficile-associated Disease.** *Vet Pathol* 2006, **43:**225–240.
14. Nielsen EO, Enoe C, Jorsal SE, Barfod K, Svensmark B, Bille Hansen V, Vigre H, Boetner A, Baekbo P: **Postweaning multisystemic wasting syndrome in Danish pig herds: productivity, clinical signs and pathology.** *Veterinary Record* 2008, **162:**505–508.
15. Brown CC, Baker DC, Barker IK: **Epithelial renewal in health and disease. Villous atrophy.** In *Pathology of Domestic Animals*, Volume 2. 5th edition. Edited by Grant Maxie M. Saunders Elsevier; 2007:77–78.
16. Morin M, Turgeon D, Jolette J, Robinson JB, Phaneuf R, Sauvageau R, Beauregard M, Teuscher E, Higgins R, Larivière S: **Neonatal diarrhoea of pigs in Quebec: Infectious causes of significant outbreaks.** *Can J Comp Med* 1983, **47:**11–17.
17. Svendsen J, Bille N, Nielsen NC, Larsen JL, Riising HJ: **Preweaning mortality in pigs. 4. diseases of the gastrointestinal tract in pigs.** *Nord Vet Med* 1975, **27:**85–101.
18. Frydendahl K: **Prevalence of serogroups and virulence genes in** ***Escherichia coli*** **associated with postweaning diarrhoea and oedema disease in pigs and a comparison of diagnostic approaches.** *Vet Microbiol* 2002, **85:**169–182.
19. Baums CG, Schotte U, Amtsberg G, Goethe R: **Diagnostic multiplex PCR for toxin genotyping of Clostridium perfringens isolates.** *Vet Microbiol* 2004, **100:**11–16.
20. Vijgen L, Moes E, Keyaerts E, Li S, Van Ranst M: **A pancoronavirus RT-PCR assay for detection of all known coronaviruses.** *Methods Mol Biol* 2008, **454:**3–12.

doi:10.1186/1746-6148-9-206
Cite this article as: Kongsted *et al.*: Microbiological, pathological and histological findings in four Danish pig herds affected by a new neonatal diarrhoea syndrome. *BMC Veterinary Research* 2013 **9**:206.

Photo: Martin Dam Kristensen

5.2 Manuscript 2

The effect of New Neonatal Porcine Diarrhoea Syndrome (NNPDS) on average daily gain and mortality in 4 Danish pig herds

Kongsted H.*, Stege H., Toft N. and Nielsen J.P.

*Corresponding author:

Hanne Kongsted, Danish Pig Research Centre

Phone: +4533394931/ +4524949736

Email: hko@lf.dk

BMC Veterinary Research

RESEARCH ARTICLE Open Access

The effect of New Neonatal Porcine Diarrhoea Syndrome (NNPDS) on average daily gain and mortality in 4 Danish pig herds

Hanne Kongsted[1,2*], Helle Stege[2], Nils Toft[3] and Jens P Nielsen[2]

Abstract

Background: The study evaluated the effect of New Neonatal Porcine Diarrhoea Syndrome (NNPDS) on average daily gain (ADG) and mortality and described the clinical manifestations in four herds suffering from the syndrome. NNPDS is a diarrhoeic syndrome affecting piglets within the first week of life, which is not caused by enterotoxigenic *Escherichia coli* (ETEC), *Clostridium perfringens* (*C. perfringens*) type A/C, *Clostridium difficile* (*C. difficile*), rotavirus A, coronavirus, *Cystoisospora suis*, *Strongyloides ransomi*, *Giardia spp* or *Cryptosporidium spp*.

Results: Piglets were estimated to have a negative ADG of 9 and 14 g when diarrhoeic for 1 day and >1 day respectively. However, if only diarrhoeic on the day of birth, no negative effect on ADG was seen. Piglets originating from severely affected litters were estimated to have a reduced ADG of 38 g. The study did not show an overall effect of diarrhoea on mortality, but herd of origin, sow parity, birth weight, and gender were significantly associated with mortality. In one of the herds, approximately 25% of the diarrhoeic piglets vs. 6% of the non-diarrhoeic piglets died, and 74% of necropsied piglets were diagnosed with enteritis. These findings indicate that the high mortality seen in this herd was due to diarrhoea.

Conclusions: NNPDS negatively affected ADG in piglets, and even piglets that were diarrhoeic for one day only experienced a reduction in ADG. However, the study showed that diarrhoea restricted to the day of birth did not affect ADG and suggested this phenomenon to be unrelated to the syndrome. Since the diarrhoeal status of the litter had important effects on ADG, future research on NNPDS probably ought to focus on piglets from severely affected litters.
The study showed important dissimilarities in the course of diarrhoea between the herds, and one herd was considerably more affected than the others. Within this herd, NNPDS seemed to be associated with a higher mortality, whereas in general the study did not show lethal effects of NNPDS.

Background

Several researchers have suggested the emergence of a new neonatal diarrhoeal syndrome [1-5]. The present study is the second in a series from our research group studying this new syndrome. All studies were carried out in the same four herds having a long history of unexplained neonatal diarrhoea. Running parallel to the current study, a microbiological study was carried out. The results of the microbiological study suggested that neither ETEC, *C. perfringens* type A/C, *C. difficile*, rotavirus A, coronavirus, *Cystoisospora suis*, *Strongyloides ransomi*, *Giardia spp* nor *Cryptosporidium spp* were associated with the syndrome [6].

Previous studies on the effect of diarrhoea in suckling pigs estimated reductions in ADG of 8–14 g per day [7,8]. Various studies have estimated significant associations between suckling pig diarrhoea and mortality [9,10] or diagnosed enteritis as an important cause of death in the suckling period [11-13].

The primary objective of the current study was to evaluate the effect of NNPDS on average daily gain (ADG) and mortality in relation to timing and duration

* Correspondence: hko@lf.dk
[1]Danish Pig Research Centre, Danish Agriculture and Food Council, Vinkelvej 13, Kjellerup 8620, Denmark
[2]HERD – Centre for Herd-oriented Education, Research and Development, Department of Large Animal Sciences, University of Copenhagen, Groennegaardsvej 2, Frederiksberg C 1870, Denmark
Full list of author information is available at the end of the article

of clinical symptoms in individual piglets. Effects were estimated with respect to individual symptoms as well as symptoms at litter level.

The secondary objective was to describe the course of disease in herds suffering from NNPDS.

Results

In total, 989 piglets were ear tagged at birth. During the study period, 110 piglets were euthanized for diagnostic purposes (for results: See [6]) and five were excluded due to hermaphroditism or missing data. This left 874 piglets (207, 228, 202 and 237 from herds 1, 2, 3 and 4, respectively) for the study on mortality.

During the study period 80 piglets died, hence could not be included in the study on ADG. Furthermore, for this part of the study a critical limit on five piglets per litter was set resulting in exclusion of 14 piglets (the remaining part of four litters). In total, 780 piglets (154, 209, 189 and 228 from herds 1, 2, 3 and 4, respectively) were evaluated in the study on ADG.

Apart from diarrhoea, only a few disease problems were recognised during the daily examinations of the herds. However, arthritis was rather prevalent in herd 4 (n = 19). Skin abrasions were the only extra-intestinal clinical symptoms sufficiently prevalent and evenly distributed between herds to be included in the statistical evaluations.

On the day of birth, approximately 20% of the piglets did not have any faeces in rectum at the time of examination. On the other days of the study absence of faeces was observed in 10% of the piglets. In both the diarrhoeic and the non-diarrhoeic piglets the colour of faeces was most often (in 70-80% of piglets) yellow. Neither debris nor blood was evident.

Table 1 presents the daily prevalence of diarrhoea within each herd. The prevalence of diarrhoea in herd 1 was high (around 30%) on each day of the study. In the other herds, the prevalence was high on the first day of life, but otherwise markedly lower than in herd 1. A total of 229 piglets (26%) were diarrhoeic on the day of birth. Within this group, 50% were diarrhoeic on the day of birth exclusively (data not shown).

Table 2 presents a descriptive summary of the diarrhoea seen in the four herds with respect to prevalence, ADG, and mortality. In herd 1, nearly half the piglets were diarrhoeic for more than one day, and the mortality among piglets being diarrhoeic (irrespective of diarrhoeal level) was approximately 25% vs. 6% among the non-diarrhoeic piglets. In the other herds, similar mortalities were seen in non-diarrhoeic piglets and piglets being diarrhoeic for one day. However, additional days of diarrhoea seemed to be associated with a higher mortality in herd 2 and herd 4. The duration of diarrhoea differed according to the parity of the sow. One third of the piglets born by first parity sows were diarrhoeic for >1 day, whereas this was observed for one fifth of the piglets born by mature sows only (data not shown).

On the fifth day of life, a total of 842 piglets were alive and examined for clinical signs of failure to thrive. Results are presented in Table 3. Signs of dehydration were only found in diarrhoeic piglets. Hollow flanks, protruding ribs, dull hair coat were more common in diarrhoeic piglets, than in pigs without diarrhoea. All clinical signs were more prevalent in pigs experiencing diarrhoea for more than one day. The general clinical picture of piglets suffering from diarrhoea was that of failure to thrive and wasting.

In total, 80 piglets (9%) died within the first ten days of life. Mortality within herds 1, 2, 3 and 4 reached 43 (21%), 15 (7%), 13 (6%) and 9 (4%), respectively. The majority of deaths in herds 2–4 occurred during the first five days of life. In herd 1, deaths were evenly distributed throughout the first ten days of life. Figure 1 shows the primary diagnoses assigned at necropsy. Within herds 1 and 4, a few cases of enteritis (9 of 32 and 3 of 3 cases, respectively) exhibited fibrino-necrotic gross lesions. The remaining cases exhibited less pronounced lesions. In addition to the diagnoses of starvation shown in Figure 1, starvation was recorded as a secondary diagnosis in a total of five piglets.

Weight gain

Results of the mixed linear model are presented in Table 4. As shown, both diarrhoea-associated variables (diarrhoea and Diarrhoeal Status of Litter (DSL)) had significant (P =

Table 1 The prevalence of diarrhoea on each day of the study within and across herds

Herd	Day 1		Day 2		Day 3		Day 4		Day 5	
	n[1]	D[2] (%)	n	D (%)	n	D (%)	n	D (%)	n	D (%)
1	207	26	202	34	201	24	197	39	194	40
2	228	41	226	23	226	13	224	11	222	15
3	202	18	200	9	198	11	195	16	193	16
4	237	19	236	9	235	8	235	6	234	7
Across herds	874	26	864	18	860	14	851	18	843	19

[1]Refers to the number of piglets alive at the day in question. [2]Diarrhoeic piglets (piglets having watery or liquid consistency of faeces).

Table 2 Descriptive data on the diarrhoea in the four herds

		Days with diarrhoea											
		None			1 day						>1 day		
					During day 1			During day 2-5					
Herd	n[1]	n	ADG (mean, sd)	Dead (%)	n	ADG (mean, sd)	Dead (%)	n	ADG (mean, sd)	Dead (%)	n	ADG (mean, sd)	Dead (%)
1	207	49	186 (83)	6	15	221 (90)	27	49	158 (80)	22	94	115 (74)	27
2	228	88	179 (65)	6	48	194 (61)	2	33	165 (60)	6	59	169 (61)	12
3	202	106	185 (60)	8	22	193 (74)	5	43	167 (57)	5	31	156 (72)	6
4	237	150	190 (62)	2	30	190 (65)	3	32	147 (61)	6	25	158 (52)	12
In total	874	393	186 (65)	5	115	196 (67)	6	157	160 (65)	11	209	145 (71)	18

[1]This number refers to the number of piglets at day 1 of the study. ADG has only been calculated for the ones surviving until day 10 of life (780 in total).

0.01 and $P < 0.001$) effects on ADG. Being part of a severely affected litter had a markedly larger effect (–38 g per day) than the individual status of the piglet (–9 - –14 g per day, depending on the duration of diarrhoea). Piglets that were only diarrhoeic on the day of birth were not negatively affected on ADG compared to the non-diarrhoeic ones. No confounding effects were recognised. The ICC estimated for the litter random effect was 42%, indicating a very large influence on weight gain by litter of origin. In pairwise comparisons, ADG in non-diarrhoeic piglets was significantly different from ADG in piglets having diarrhoea for >1 day. Also, ADG in piglets only being diarrhoeic at the day of birth was significantly different from ADG in piglets having diarrhoea for >1 day. Data were insufficient to show other statistically significant differences.

Mortality

Results of the generalized linear mixed model are presented in Table 5. None of the diarrhoea-associated variables (diarrhoea or DSL) were kept in the final model, indicating that diarrhoea was not a significant risk factor with respect to mortality, when other variables and random litter effects were taken into account. Herd of origin was the most important risk factor in the model. In herd 1, odds for dying were significantly higher than in the other herds and almost twelve times as high as in herd 4, which had the lowest odds for dying. No confounding effects were recognised. In pairwise comparisons, herd 1 was the only herd significantly different from the others.

Table 3 Clinical signs of failure to thrive in 842 piglets on day five of life

	Days with diarrhoea			
Clinical finding[1]	None	1 day		>1 day
		During day 1	During day 2-5	
Hollow flanks	18%	10%	23%	38%
Protruding ribs	3%	2%	9%	22%
Dull hair coat	38%	27%	41%	50%
Dehydration	0%	0%	3%	15%

[1]Definitions used: Hollow flanks: The area behind the ribs deviated inwards; Protruding ribs: Ribs and spine were visible; Dull hair coat: Hair coat appeared dull and longer than normal; Dehydration: sunken eye-balls and lack of skin-elasticity.

Discussion

The study monitored the daily faecal consistency in individual neonatal piglets, and therefore offered an opportunity to thoroughly evaluate prevalence, timing and duration of NNPDS, and its effect on piglets. The observational character of the study was, however, affected by the removal of 110 piglets for diagnostic purposes. This interference, which potentially increased ADG and decreased mortality in litters supplying piglets for diagnostic purposes, was addressed by inserting a random effect of litter in the statistical models. Altogether, we considered that taking out piglets for laboratory examination

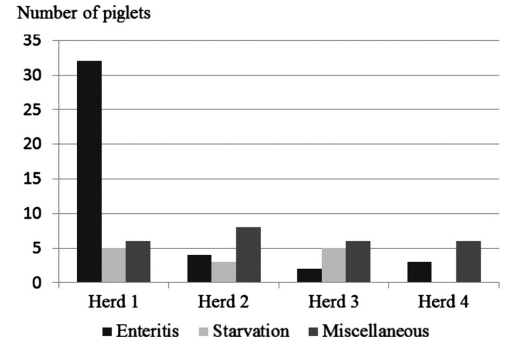


Figure 1 Primary diagnoses at necropsy in 43, 15, 13 and 9 piglets from herd 1, 2, 3 and 4, respectively. The following criteria were used to assign piglets to the respective diagnoses: Enteritis: Intestinal hyperaemia, flaccidity or mucosal lesions and liquid contents in colon. Starvation: Piglets were emaciated and stomachs and intestines were empty. Miscellaneous: All other diagnoses and cases without obvious findings.

Table 4 Final linear mixed model on ADG in 780 piglets from the four herds

Risk factor	Estimate (g/day)	SE (g/day)	P-value
Intercept (1000 g birth weight)	151	7	
Parity			0.7[1]
Mature	0		
Young	5	11	
Birth weight (per 100 g increase)	9	0.6	< 0.001
Diarrhoea[2]			0.01
None	0[a]		
1 day (during day 1)	4[a]	6	
1 day (during day 2–5)	−9[ab]	5	
>1 day	−14[b]	5	
Diarrhoeal status of litter			< 0.001
Mildly affected	0		
Severely affected	−38	11	
Random effect litter[3]	42%		

[1]Parity was kept in the model due to its biological importance.
[2]Levels of diarrhoea with different letters as superscript are significantly different ($\alpha < 0.05$) when compared pairwise.
[3]The random effect of litter is displayed as the Intraclass Correlation Coefficient (ICC).

Table 5 Results of the final generalised mixed model on mortality during the first 10 days of life in 874 piglets from the four herds

Risk factor	Coefficient	SE	OR[1]	P-value
Intercept (1000 g birth weight)	−3.9			
Herd[2]				< 0.001
Herd 4	0[a]			
Herd 3	1.0[a]	0.7	2.3	
Herd 2	0.5[a]	0.7	1.3	
Herd 1	2.7[b]	0.6	11.8	
Parity				0.06[3]
2nd-7th	0			
1st	0.8	0.4	1.8	
Birth weight (per 100 g increase)	−0.3	0.06	0.6	<0.001
Gender				0.001
Female	0			
Male	0.9	0.3	2.1	
Random effect of litter[4]	29%			

[1]The OR presented is the population average OR, and relates to an average piglet in any litter of the study.
[2]Herds with different letters as superscript are significantly different ($\alpha < 0.05$) when compared pairwise.
[3]Parity was kept in the model due to its biological significance.
[4]The random effect of litter is displayed as the Intraclass Correlation Coefficient (ICC).

during the course of the study was the best way to obtain a microbiological diagnosis of the existing clinical problems.

The procedure of adjusting litters to only 11 or 12 piglets and excluding the smallest piglets aimed at avoiding insufficiency of colostrum and milk. Milk-filled stomachs at necropsy generally confirmed, that diarrhoea due to starvation was rare during the period of investigation. Under normal field conditions, starvation is likely to be more prevalent and to interfere with the clinical picture of the syndrome to a higher extent.

Antibiotic treatment was allowed due to ethical concerns but potentially influenced the results of the study. However, since the syndrome seems to be characterised by non-responsiveness to antibiotics (personal communication S.E. Jorsal, National Veterinary Institute, Technical University of Denmark) the beneficial effects of antibiotics on ADG and mortality were probably relatively small.

A relatively large group of piglets (10-20%) did not have faeces in rectum at the daily examinations and consequently were recorded as non-diarrhoeic. Therefore the daily prevalence of diarrhoea (ranging from 6% to 40%) might have been underestimated. For the analysis, this was not considered a major problem since diarrhoeal status of each piglet was evaluated for the total study period.

Clinical signs of failure to thrive were overt in piglets being diarrhoeic for >1 day, and in some cases even 1 day of diarrhoea resulted in hollow flanks, protruding ribs and signs of dehydration. These findings match experiences from swine practitioners, that NNPDs is a debilitating syndrome, significantly affecting the well-being of piglets (personal communication, S.E. Jorsal).

The negative effects of diarrhoea estimated in this study were comparable to the −8 g per day estimated in a previous study involving the whole suckling period [8]. In the current study, even piglets only being diarrhoeic for a single day (if this was not the day of birth) had a reduced ADG of 9 g compared to non-diarrhoeic piglets. The measurable (though not statistically significant) effect of a single day with diarrhoea underlines the severity of the syndrome.

Interestingly, many piglets (26%) were diarrhoeic on the day of birth and those (50%) that were only diarrhoeic on the day of birth were not negatively affected on ADG. Apparently, many cases of diarrhoea on the day of birth were unrelated to disease.

The large effect of DSL pointed out that the diarrhoea seen on the level of litters was the most important kind of diarrhoea in terms of disease. ADG was not affected by herd of origin, but was heavily influenced by litter of origin (ICC = 42%). A large litter effect was not surprising since it comprised both issues related to the study design (as discussed earlier) and all factors related to the

performance of the individual sows. Lack of herd effect on ADG was in accordance with the study by Johansen et al. [8].

Compared to the study by Svensmark et al. [7], which estimated the effect of diarrhoea at the litter level to be −14 g per day, the litter-related estimate of −38 g in this study was more pronounced. Possibly, NNPDS affected the piglets more violently than the diarrhoea seen in the previous study. However, the estimates from the two studies were not directly comparable, since the former included the whole suckling period and relied on farmers registrations of diarrhoea.

Regarding mortality, herd was the most important risk factor, with odds for dying in herd 1 being 5–12 times higher than in the other herds. Descriptive data indicated that the excess mortality seen in this herd was caused by diarrhoea, since the mortality among diarrhoeic piglets, irrespective of the duration of diarrhoea, was four times higher than the mortality among non-diarrhoeic ones. In the other herds, mortalities were lower at all levels of diarrhoea, and any association between diarrhoea and mortality was less obvious.

Suckling piglet diarrhoea has often been associated with mortality. In a previous study involving 3600 piglets from a single herd, neonatal diarrhoea was estimated to increase odds of dying by 2.7 [9]. Another study involving piglets from 70 herds showed that diarrhoeic litters had increased losses during the suckling period of 0.8 piglets [10]. Furthermore, different studies have diagnosed enteritis as the primary cause of death in 4-14% cases of suckling pig mortality [11-13].

In the current study, we did not find an effect of NNPDS on mortality when herd of origin and other risk factors were taken into account. The underlying aetiology of diarrhoea in the study by Gardner et al. [9] was not investigated, and discrepancies could perhaps be explained by differences in aetiology. Herd effects were not considered in the results presented by Lingaas et al. [10], however, may have explained some of the losses attributed to diarrhoea. Overall, it is important to notice that the generally low mortality in the current study was probably partly due to study design issues. Despite these limitations, the study indicated that NNPDS was generally not associated with high mortality. This finding matched herd experiences as reported by swine practitioners [14].

Conclusions

The diarrhoeas observed within herds suffering from NNPDS were yellow with no evidence of blood or debris. Three of the four herds had similar courses of NNPDS. In these herds the daily prevalence of diarrhoea was approximately 15%, deaths occurred within the first five days of life, and the mortality was generally low. The most prevalent diagnoses assigned at necropsy were miscellaneous and starvation. In the last herd (herd 1), diarrhoea affected more piglets, and many piglets were affected for a longer period of time. In this herd, a high mortality was seen during the whole study period, and enteritis was the most prevalent diagnosis at necropsy.

Overall, the study showed that NNPDS severely affected the well-being in piglets and reduced the ADG. Data were not sufficient to estimate a significant effect of diarrhoea for a single day, however, a tendency was clear, and this finding underlines the severity of the condition. Diarrhoea restricted to the day of birth was common and did not affect ADG. Thus, the study suggested this phenomenon to be unrelated to the syndrome.

Since the diarrhoeal status of the litter had important effects on ADG, future research on NNPDS should probably focus on piglets in severely affected litters.

Overall, the study demonstrated that NNPDS was not associated with mortality. In the herd associated with the highest odds for mortality, however, the increased mortality appeared to be due to diarrhoea.

Methods

Study design and inclusion of herds

The study was an observational cross-sectional study with follow-up, carried out in four Danish sow herds. Herds were included based on five criteria; problems with diarrhoea during the first week of life with a poor response to antibiotics (≥30% diarrhoeic litters for a period of ≥ 6 months), routine vaccination of sows against *E. coli* and *C. perfringens* type C, failure of preventive management interventions as verified by the local veterinarian, a PRRS negative farrowing unit as demonstrated in blood samples and negative results of routine diagnostic examinations for ETEC, *C. perfringens* type C and rotavirus A in five diarrhoeic piglets aged one to four days. During the study period, approximately 30 piglets per herd were taken out for microbiological examination. The examinations carried out indicated that the herds did not suffer from known infectious causes of diarrhoea and therefore supported a diagnosis of NNPDS [6].

Practical setup in the herds

Litters (approximately 20 litters from one farrowing batch per herd) were standardized to 11 or 12 piglets by simple random sampling among littermates with a minimum birth weight of 800 grams. No cross-fostering was allowed.

As a general rule no antibiotics were given on the first two days of life. In herd 4, however, eight pigs were treated on the second day of life due to arthritis. From the third day of life and onwards, diseases were treated with antibiotics according to individual herd routines. As a result of this, most of the diarrhoeic piglets in the

study were medicated. Non-antibiotic oral supplements were allowed to be used according to individual herd routines. In herd 2 and 4 a milk formula was given to approximately 10 piglets per herd for one or two days. In herd 1 and 3, no oral supplements were used.

Examination of piglets

Piglets were weighed at birth and at ten days of life. In each piglet rectal swabs were evaluated on a daily basis from the day of birth and five days forwards. On each evaluation, consistency of faeces was categorized into one of six categories; No faeces present (the rectal swab was dry and clean), watery, liquid, creamy, firm or solid (solid bulbs on the rectal swab). For the analyses, a piglet was defined as diarrhoeic on a particular day if having watery or liquid consistency of faeces. Microbiological testing was not performed on piglets in the study.

During the first five days of life, registrations on arthritis, respiratory disease, CNS-related disease and skin abrasions on head and fore-knees were carried out. Piglets were registered with the respective diagnoses if registered on any day. The presence of hollow flanks, protruding ribs, dull hair coats and dehydration (sunken eye-balls and loss of skin-elasticity) was registered on the fifth day of life as clinical signs of failure to thrive due to diarrhoea.

During the 10 day study period, all deaths were individually recorded and necropsies were performed on all piglets dying. At necropsy, piglets were assigned to one of three primary diagnoses; enteritis, starvation or miscellaneous. A diagnosis of enteritis was assigned if hyperaemia, flaccidity or mucosal lesions were seen in the intestines and contents of colon were liquid. A diagnosis of starvation was assigned if the piglet was emaciated, stomach and intestines were empty and no other findings were obvious. Cases of crushing, constipation, castration injuries as well as cases with no obvious lesions were assigned to the miscellaneous category. In cases when empty stomachs were seen in association with other lesions, starvation was assigned as a secondary diagnosis.

All procedures in the herds were carried out by the corresponding author.

Description of variables

Outcome variables

Weight gain (measured in g per day) and death (yes/no) during the period from birth until ten days of age, were the dependent variables in the two models of the study.

Explanatory variables

The explanatory variable of primary interest was diarrhoea which was initially individually recorded on a daily basis. Subsequently, based on the duration and timing, four levels of diarrhoea were defined; "None", "1 day (during day 1)", "1 day (during day 2–5) and ">1 day" (see Table 6). These levels were introduced in order to be able to distinguish between diarrhoea at the day of birth and diarrhoea of different duration. On the litter-level, the variable "Diarrhoeal Status of Litter (DSL)" was introduced, in order to dichotomise litters into mildly affected litters (<50% diarrhoeic piglets) vs. severely affected litters (≥50% diarrhoeic piglets). Table 6 presents all explanatory variables addressed in the statistical models of the study.

Statistical analyses

For the study on weight gain, a linear mixed-effect model was used, whereas a generalised linear mixed-effect model was used in the study on mortality. Both models were fit in R [15] using the lme4 package [16]. The assumption of

Table 6 Explanatory variables addressed in the models on ADG and mortality

Explanatory variable	Level	Interpretation of levels
Primary		
Diarrhoea	None	The piglet was not diarrhoeic at any day
	1 day (during day 1)	The piglet was diarrhoeic for one day - at the day of birth
	1 day (during day 2–5)	The piglet was diarrhoeic for one day during the second to fifth day of life
	> 1 day	The piglet was diarrhoeic for more than one day during the five day study-period
Secondary		
Litter level		
Parity	Young	1st parity
	Mature	2nd – 7th parity
Diarrhoeal status of litter (DSL)	Severely affected	50% or more of the piglets in the litter were diarrhoeic for one or more days (the day of birth did not count)
	Mildly affected	Less than 50% of the piglets of the litter were diarrhoeic for one or more days (the day of birth did not count)
Piglet level		
Gender	Male	
	Female	
Birth weight	Continuous scale	
Skin abrasions	Yes	Skin abrasions on head or fore-knees at any point during the study period
	No	No skin abrasions on head or fore-knees
Herd effect	1	Effect of being born in herd 1
	2	Effect of being born in herd 2
	3	Effect of being born in herd 3
	4	Effect of being born in herd 4

linearity in the linear mixed model was verified by visual inspection of the xy-plot of ADG against birth weight. The linearity of birth weight at the log odds scale for mortality in the generalised linear mixed model was assessed by transforming birth weight into a categorical variable based on quartiles and then verify a decreasing trend of the estimates for the levels of the categorical variable.

In the models, herd of origin was included as a fixed effect, since we were interested in the specific effect of each herd. Litter of origin was included as a random effect to correct for clustering within litters. All secondary risk factors and all possible two-way interaction terms with diarrhoea were included in the initial models. Model reduction was carried out using stepwise backwards elimination, removing variables with $p > 0.05$. Since parity was considered of overall biological importance, this variable was forced into the models. Confounding was assessed by re-entering variables into the final models and checking if estimates for diarrhoea changed. Pairwise post-hoc comparisons within significant variables with more than two levels were carried out using the lsmeans package in R [17].

Ethical approval

The study was conducted in accordance with the guidelines of the Danish Ministry of Justice with respect to animal experimentation and care of animals under study. Clinical examinations and weighing were carried out with consideration to the welfare of the pigs by a skilled person (HK).

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

All authors contributed to the design of the study. Inclusion of herds, clinical examination in the herds and statistical analyses were performed by HK. All authors participated in drafting the manuscript and proofreading of the final manuscript. All authors read and approved the final manuscript.

Acknowledgements

The authors wish to thank herd-owners and their staff for help and collaboration, as well as Amalie Bækbo, Mikkel Haugegaard, Lars Jensen and Camilla Bjørn Olesen for assisting in the herds.

Author details

[1]Danish Pig Research Centre, Danish Agriculture and Food Council, Vinkelvej 13, Kjellerup 8620, Denmark. [2]HERD – Centre for Herd-oriented Education, Research and Development, Department of Large Animal Sciences, University of Copenhagen, Groennegaardsvej 2, Frederiksberg C 1870, Denmark. [3]National Veterinary Institute, Technical University of Denmark, Bülowsvej 27, Frederiksberg C 1870, Denmark.

Received: 22 October 2013 Accepted: 16 April 2014
Published: 22 April 2014

References

1. Svensmark S: **New Neonatal Diarrhoea Syndrome in Denmark**. In *Proceedings of the 1st ESPHM*. Edited by Stege H, Kristensen CS. Copenhagen, Denmark: Faculty of Life Sciences, University of Copenhagen; 2009:27.
2. Astrup P: **Neonatal Diarrhoea in Danish pig Farms - a Questionnaire Among Veterinarians.** In *Proceedings of the 21st IPVS Congres.* Edited by D'Allaire S, Friendship R. Canada: Vancouver, British Columbia; 2010:751.
3. Melin L, Wallgren P, Mattson S, Stampe M, Löfstedt M: **Neonatal Diarrhoeain Piglets from E. Coli Vaccinated Sows in Sweden.** In *Proceedings of the21st IPVS Congres.* Edited by D'Allaire S, Friendship R. Canada: Vancouver, British Columbia; 2010:290.
4. Gin T, Le Guennec J, Morvan H, Martineau G-P: **Clinical and Laboratory Investigations in 10 French pig Herds Dealing With Enzootic Neonatal Diarrhoea.** In *Proceedings of the 21st IPVS Congres.* Edited by D'Allaire S, Friendship R. Canada: Vancouver, British Columbia; 2010:758.
5. Wallgren P, Mattson S, Merza M: **New Neonatal Porcine Diarrhoea. II. Aspects on Etiology.** In *Proceedings of the 22nd IPVS Congres.* Jeju, Korea; 2012:76.
6. Kongsted H, Jonach B, Haugegaard S, Angen O, Jorsal SE, Kokotovic B, Larsen LE, Jensen TK, Nielsen JP: **Microbiological, pathological and histological findings in four Danish pig herds affected by a new neonatal diarrhoea syndrome.** *BMC Vet Res* 2013, **9**:206.
7. Svensmark B, Jorsal SE, Nielsen K, Willeberg P: **Epidemiological studies of piglet diarrhoea in intensively managed Danish sow herds. I. Pre-weaning diarrhoea.** *Acta Vet Scand* 1989, **30**(1):43–53.
8. Johansen M, Alban L, Kjaersgard HD, Baekbo P: **Factors associated with suckling piglet average daily gain.** *Prev Vet Med* 2004, **63**(1/2):91–102.
9. Gardner IA, Hird DW, Franti CE: **Neonatal survival in swine: effects of low birth weight and clinical disease.** *Am J Vet Res* 1989, **50**(5):792–797.
10. Lingaas F: **Epidemiological and genetical studies in Norwegian pig herds. IV. Breed effects, recurrence of disease, and relationship between disease and some performance traits.** *Acta Vet Scand* 1991, **32**(1):107–114.
11. Christensen J, Svensmark B: **Evaluation of producer-recorded causes of preweaning mortality in Danish sow herds.** *Prev Vet Med* 1997, **32**(3–4):155–164.
12. Vaillancourt JP, Stein TE, Marsh WE, Leman AD, Dial GD: **Validation of producer-recorded causes of preweaning mortality in swine.** *Prev Vet Med* 1990, **10**(1–2):119–130.
13. KilBride AL, Mendl M, Statham P, Held S, Harris M, Cooper S, Green LE: **A cohort study of preweaning piglet mortality and farrowing accommodation on 112 commercial pig farms in England.** *Prev Vet Med* 2012, **104**(3–4):281–291.
14. Kongsted H: **Spædgrisediarré i Danmark anno 2013 (Danish).** http://vsp.lf.dk/~/media/Files/PDF%20-%20Publikationer/Erfaringer%202013/Erfaring_1320_Sp%C3%A6dgrisediarre_i_Danmark_anno_2013.ashx.
15. CoreTeam R: *R: A Language and Environment for Statistical Computing.* Vienna, Austria: R foundation for statistical computing; 2013. http://WWW.R-project.org/.
16. Bates D, Maechler M, Bolker B: **lme4: Linear mixed-effects models using S4 classes.** 2013, http://CRAN.R-project.org/package=lme4.
17. Lenth RV: **lsmeans: least-squares means.** 2013, http://CRAN.R-project.org/package=lsmeans.

doi:10.1186/1746-6148-10-90
Cite this article as: Kongsted *et al.*: The effect of New Neonatal Porcine Diarrhoea Syndrome (NNPDS) on average daily gain and mortality in 4 Danish pig herds. *BMC Veterinary Research* 2014 **10**:90.

Photo: Martin Dam Kristensen

5.3 Manuscript 3

Risk factors and epidemiological characteristics of new neonatal porcine diarrhoea syndrome in four Danish herds.

Kongsted H.*, Toft N. and Nielsen J.P.

*Corresponding author:

Hanne Kongsted, Danish Pig Research Centre

Phone: +4533394931/ +4524949736

Email: hko@lf.dk

BMC Veterinary Research

RESEARCH ARTICLE Open Access

Risk factors and epidemiological characteristics of new neonatal porcine diarrhoea syndrome in four Danish herds

Hanne Kongsted[1,2*], Nils Toft[3] and Jens Peter Nielsen[2]

Abstract

Background: The epidemiology of New Neonatal Porcine Diarrhoea Syndrome (NNPDS) was studied in four selected herds. A total of 941 new born piglets in 86 litters were evaluated for five consecutive days. NNPDS is a newly emerged syndrome, characterized by diarrhoea within the first week of life, which is un-responsive to antibiotics and not associated with known pathogens. The aetiology behind the syndrome is unknown, and specific risk factors predisposing piglets to develop NNPDS also remain to be determined.
The study evaluated sow and piglet-level risk factors for developing NNPDS and described the epidemiologic characteristics within four herds previously diagnosed with the syndrome. NNPDS was defined as diarrhoea at any time-point during the second to fifth day of life.

Results: NNPDS was observed in a total of 60% (range: 39%-89%) of first parity piglets and 36% (range: 19-65%) of piglets born by mature sows. In total of 26% of piglets had liquid faeces on the day of birth. Approximately half of these piglets developed NNPDS. In the majority of cases (50-70% of cases within herds) symptoms started on the second or third day of life. Piglets in Herd 1 had12.8 times higher probability of developing NNPDS than piglets in Herd 4. First parity piglets had a 4.1 higher probability of developing NNPDS than piglets born by mature sows. Birth weight and faecal consistency on the day of birth were minor risk factors, each significant within one herd.

Conclusions: The most important factors associated with NNPDS were herd of origin and sow-parity. The reason for one of the herds experiencing a considerably more severe outbreak than the others was not explained by factors addressed in this study.
The epidemiological pattern of diarrhoea varied a lot between herds; however, in all herds first parity piglets seemed predisposed. This association may be explained by an infectious background of the syndrome, but further studies are needed to explain this association.

Background

Neonatal diarrhoea is a well-known disease complex in modern swine production influenced by individual, maternal and environmental factors. The aetiology in specific herd-cases may differ and is often incompletely diagnosed. Until recently, this complexity was not of major practical concern, since most problems could be controlled by vaccination or antibiotics. Around 2008, however, a new syndrome that did not respond to antibiotics or commercial vaccines seemed to emerge ([1] + Personal communications, S.E. Jorsal, National Veterinary Institute, Technical University of Denmark and B. Svensmark, Pig Research Centre, Danish Agriculture & Food Council, Denmark). The number of affected herds is unknown, but 80% of Danish swine practitioners report to experience these problems [1].

Currently, New neonatal porcine diarrhoea syndrome (NNPDS) refers to a clinical picture with piglets developing diarrhoea that is un-responsive to antibiotics within the first days of life. The suggested piglet-level case-definition is; Non-haemorrhagic diarrhoea during the first week of life without detection of known infectious

* Correspondence: hko@lf.dk
[1]Danish Pig Research Centre, Danish Agriculture and Food Council, Vinkelvej 13, 8620 Kjellerup, Denmark
[2]Department of Large Animal Sciences, HERD – Centre for Herd-oriented Education, Research and Development, University of Copenhagen, Groennegaardsvej 2,1870 Frederiksberg C, Denmark
Full list of author information is available at the end of the article

pathogens, which is characterized by milk-filled stomachs and flaccid intestines at necropsy [2]. This definition is based upon diagnostic examination of 101 Case and Control piglets from the four herds of the current study. Infectious agents which were evaluated and considered not to be involved in the syndrome included; Enterotoxigenic *E. coli*, *Clostridium perfringens* type A and C, *Clostridium difficile*, rotavirus A, coronavirus, *Cystoisospora suis*, *Strongyloides ransomi*, *Giardia spp* and *Cryptosporidium spp*.

Specific factors predisposing piglets to develop NNPDS remain to be discovered. Health problems in sows have previously been associated with diarrhoea in suckling pigs [3-5], but veterinary practitioners do not seem to associate the mastitis-metritis-agalactiae syndrome (MMA) or other sow health conditions with NNPDS [1]. Practitioners report on an association with first parity sows, [1], but this experience needs scientific evaluation.

Insufficient prenatal nutrition or inadequate colostrum supplies are well-known risk factors for neonatal diarrhoea, thus clinical signs suggesting such problems need evaluation in outbreaks of NNPDS. A previous study showed that liquid faeces on the day of birth did not have any negative effects on piglets in these herds in terms of weight gain. Therefore, it was hypothesized that liquid faeces at birth might be a normal phenomenon, unrelated to the syndrome [6]. One of the aims of the present study was to elaborate on this hypothesis, by evaluating if liquid faecal consistency on the day of birth was associated with diarrhoea on the subsequent days.

The primary aim of the study was to investigate sow- and piglet-level risk factors associated with NNPDS. Furthermore, the epidemiological pattern of diarrhoea in terms of prevalence, timing, duration and tendency to cluster within litters was described within the separate herds of the study. Since day one was hypothesized not to be part of the syndrome and piglets were only evaluated until the fifth day of life, NNPDS was defined as diarrhoea at any point during the second to fifth day of life.

Results

Description of herds

The four study-herds were conventional indoor production herds and the piglets under study were all Landrace-Yorkshire-Duroc cross-breeds. All herds had experienced problems with neonatal diarrhoea for at least one year. None of the herd-owners were able to point out changes in management connected with the outbreaks. All herds had weekly farrowings and practiced all- in/all-out in farrowing units with appropriate cleaning between farrowing batches. Farrowing crates had partially slatted floors with supplemental heat and cover provided for the piglets. Herd details are given in Table 1.

Data structure and important herd differences

Altogether, 989 piglets within 86 litters were included at birth. A total of 48 piglets were removed from the data because they were euthanized for necropsy with no history of NNPDS (n = 27), died (n = 20) prior to day five with no history of NNPDS or were hermaphroditic (n = 1). Thus, a total of 941 piglets (227, 245, 216 and 253 from Herds 1, 2, 3 and 4) were included in the analyses. Within Herds 1–4, 5, 10, 5 and 9 first parity sows and 17, 11, 16 and 13 mature sows (2nd-7th parity) were included.

Table 1 Characteristics of the four study-herds

Herd data	Herd 1	Herd 2	Herd 3	Herd 4
Study period	January 2011	March 2011	May 2011	July 2011
Duration of problems	2 years	>1 year	Since establishment (2 years)	2 years
Herd size (n sows)	900	1250	700	950
Sows per farrowing room	27	40	42	44
SPF[1]-status	Not declared	Not declared	SPF + AP12	SPF
Piglets weaned/sow/year[2]	30.7	27.1	25.4	32.3
1st parity litters (%)[2]	20	22	21	23
Recruitment of gilts	Purchase	Own production	Purchase	Own production
Semen	Purchase	Own boars	Own boars	Purchase
Sow feed[3]	Liquid (residue-free)/Home made	Liquid (residue-free)/ Home made	Liquid (residue-free)/ Home made	Liquid/Factory made
Routine treatment of piglets[4]	None	None	Amoxicillin at birth	Amoxicillin at castration
Routine treatment of sows[5]	None	None	Oxytocin after farrowing	Oxytocin after farrowing

[1]Specific Pathogen Free – In Denmark most herds participate in a surveillance programme, and are registered for freedom/presence of certain infectious diseases, including PRRS, *M. hyopneumoniae, A. pleuropneumoniae, P. multocida* tox + and *B. hyodysenteriae*. SPF + Ap12 means that the herd is declared free from all SPF diseases except *A. pleuropneumoniae* type 12. [2]Average calculated from herd-registrations made in a 3 month period prior to investigation. [3]Feed type used in farrowing period. [4]Standard antibiotic treatments used in the herds during the first week of life. These treatments were not used during the study.
[5]Any standard medication used on the day of parturition. These treatments were also used during the study.

In Herds 1–3, 10% of sows were treated with antibiotics and NSAIDs on day one, whereas this counted for 41% (78% of first parity sows and 15% of mature sows) in Herd 4. All sows in Herd 3 and Herd 4 were treated with oxytocin postpartum. In Herd 2 and Herd 4 a milk formula was given to piglets by drench for supportive care (approximately 10 piglets per herd). Despite the general rule of no antibiotic treatment prior to day 3, in Herd 4, a total of 13 piglets were treated with streptocillin on the second day of life due to arthritis. These piglets were kept in the study, since the treatment was considered of minor importance in the context.

Clinical findings in piglets on the first day of life

Hollow flanks, rough hair coats, perineal staining and liquid consistency of faeces were relatively prevalent findings in all herds. Liquid consistency of faeces was seen in a total of 26% of piglets (30% of first parity piglets and 23% of piglets born by mature sows). Protruding ribs, fore-knee abrasions and dehydration were low prevalent findings in all herds. A correlation ($r = 0.67$) between faecal consistency and perineal staining was seen. Therefore only faecal consistency was evaluated in the risk-factor analyses. Table 2 presents day one clinical findings in piglets. First parity piglets in Herd 2 were lighter than first parity piglets in the other herds. Furthermore, piglets in this herd had the highest prevalence of hollow flanks, rough hair coats, perineal staining and liquid consistency of faeces.

Clinical findings in sows on the day of parturition

Results of sow examinations are summarized in Table 3. Mature sows in Herds 2 and 3 had smaller litter sizes than mature sows in Herd 1 and 4 (mean size approximately 18 piglets vs. 20 piglets). Litter sizes of first parity sows where alike across herds (mean size approximately 15 piglets). The majority of the sows (63/86) did not have any obvious health problems. Certain differences were seen between herds; Herd 1 having more sows suffering from fever and leg problems, Herd 2 having more sows with vulva discharge and Herd 3 having the highest prevalence of clinical mastitis. No obvious link between clinical registrations (made by the first-author of the manuscript) and treatment (carried out by staff-persons) was seen. First parity sows in Herd 4 were very often treated compared to the remaining sows in the study. The staff-persons indication to treat was mastitis.

Occurrence of NNPDS (diarrhoea at day 2–5 of life)

In total, 198 (60%) first parity piglets and 221 (36%) of piglets born by mature sows were diarrhoeic at some point between day 2 and five, thus were classified as suffering from NNPDS. The within-herd prevalence of NNPDS and associations between liquid faeces on day one and NNPDS are presented in Table 4. Out of 241 piglets having liquid faeces on the day of birth, a total of 130 (54%) developed NNPDS.

In the majority of cases (50-70% of cases within herds), symptoms started on the second or third day of life (Figure 1). The duration of NNPDS (the number of diarrhoeic days between day two and five) in piglets within

Table 2 Day one clinical findings in 941 piglets from the four herds

Herd	1	2	3	4	Total
Piglets (first parity/mature sows)	54/173	117/128	53/163	104/149	328/613
Birth weight (kg) Mean (sd)					
First parity piglets[1]	1.36 (0.3)[a]	1.26 (0.2)[b]	1.34 (0.3)[ab]	1.33 (0.2)[a]	1.31 (0.2)
Piglets born by mature sows[1]	1.44 (0.3)[a]	1.42 (0.3)[a]	1.47 (0.3)[a]	1.42 (0.3)[a]	1.44 (0.3)
Clinical appearance (parities combined)					
Hollow flanks	28%	52%	30%	48%	40%
Rough hair coat	16%	53%	48%	39%	39%
Perineal staining[2]	33%	55%	29%	22%	33%
Liquid faeces[2]	25%	40%	18%	19%	26%
Protruding ribs	3%	3%	0.5%	1%	2%
Fore-knee abrasions	2%	0.4%	0.5%	5%	2%
Dehydration	0%	0%	0%	0%	0%
Clinical signs of failure to thrive[3]					
First parity piglets	46%	89%	88%	77%	77%
Piglets born by mature sows	51%	78%	58%	74%	64%

[1]Different letters within rows indicate significant ($P > 0.05$) in Welch *t*-test.
[2]Since these variables were correlated, only faecal consistency was evaluated in the risk-factor analysis.
[3]One or more of the following clinical signs; hollow flanks, rough hair coat, perineal staining, liquid faeces, protruding ribs, fore-knee abrasions and dehydration.

Table 3 Litter sizes, clinical registrations and medical treatment on the day of parturition in 86 sows within the four herds

Herd	1	2	3	4	Total
n (first parity/Mature)	5/17	10/11	5/16	9/13	29/57
Litter size Mean (sd)	18.6 (2.6)	16.2 (2.8)	17.3 (2.4)	18.3 (3.6)	17.6 (3)
Litter size 1st parity[1]	15.6 (1.1)[a]	14.4 (2)[a]	16.2 (2.4)[a]	15.8 (2)[a]	15.3 (2)[2]
Litter size mature sows[1]	19.5 (2.2)[a]	17.9 (2.3)[ab]	17.6 (2.3)[b]	20.1 (3.4)[a]	18.8 (2.7)[2]
Stillborn Mean (sd)	2 (1.6)	1 (1.14)	1.7 (1.6)	1.3 (1.3)	1.5 (1.5)
Fever (≥39.5°C)	6 (27%)	3 (14%)	2 (10%)	2 (9%)	13 (15%)
Leg problems	5 (23%)	1 (5%)	0 (0%)	2 (9%)	8 (9%)
Mastitis	0 (0%)	0 (0%)	4 (19%)	1 (5%)	5 (6%)
Vulva discharge	0 (0%)	2 (10%)	0 (0%)	0 (0%)	2 (2%)
Medication[2,3]	2 (9%)	2 (10%)	3 (14%)	9 (41%)	16 (19%)

[1]Different letters within rows indicate significant (P > 0.05) in Welch *t*-test.
[2]Medication included antibiotics and NSAIDs (in Herd 3, two of the sows were treated with NSAIDs only).
[3]Within Herds 1–3, both first parity sows and mature sows were treated. In Herd 4, seven first parity sows (78%) and two mature sows (15%) were treated.

the four herds is presented in Figure 2. Being affected for one or two days seemed to be the norm, but a few first parity piglets and piglets within Herd 1 experienced symptoms for a longer period. The within-litter prevalence of NNPDS is presented in Figure 3. Both first parity litters and litters of mature sows were affected, but first parity litters were constantly affected and generally had a larger number of diarrhoeic on piglets. In affected first parity litters in average 60% (range: 34-88% in separate herds) of piglets suffered from NNPDS. This counted for an average of 38% (range: 17-62% within separate herds) of piglets in mature parity litters.

Apart from the tendency to affect first parity litters most, no obvious pattern was seen across herds. In Herd 1, piglets with NNPDS seemed to be clustered in litters, whereas in Herd 3 and 4, piglets with NNPDS seemed to be more evenly distributed among litters. In Herd 2, a strong tendency for NNPDS to cluster in first parity litters was observed. Herd 4 stood out as the least affected herd – half of the litters in this herd were either unaffected or had a single piglet with NNPDS only.

Risk factors for NNPDS at sow level

In the separate parity models (step 1 of the statistical analysis), none of the sow-effects came out significant. Thus, neither litter size, stillborn piglets nor clinical disease in sows were significant risk factors for NNPDS.

The final overall model (step 2 of the statistical analysis) is presented in Table 5. Herd of origin was the most important factor associated with the development of NNPDS, with an OR_{PA} of 12.8 in piglets from Herd 1 compared to piglets from Herd 4. Parity was also an important risk factor with an OR_{PA} of 4.1 in first parity piglets compared to mature parity piglets.

Risk factors for NNPDS at piglet level

Herd-specific models including the variables that were significant in the overall model were constructed (step 3 in the statistical analysis). Results are presented in Table 6. Parity was the only significant risk factor for NNPDS within all herds (OR_{PA} between 2.4 and 7.9). Random litter effects differed a lot between herds (ICC between 1% (Herd 3) and 39% (Herd1)). Birth weight and faecal

Table 4 Prevalence of liquid faecal consistency day one and NNPDS in first parity piglets and piglets born by mature sows in the four herds

Herd	1	2	3	4	Total
First parity piglets (n)	54	117	53	104	328
Liquid faeces day one	12 (22%)	52 (44%)	10 (19%)	24 (23%)	98 (30%)
NNPDS[1]	48 (89%)	74 (63%)	35 (66%)	41 (39%)	198 (60%)
NNPDS/Liquid faeces day one[2]	10/12	39/52	5/10	12/24	66/98
Piglets born by mature sows (n)	173	128	163	149	613
Liquid faeces day one	45 (26%)	47 (37%)	28 (17%)	23 (15%)	143 (23%)
NNPDS[1]	112 (65%)	28 (22%)	53 (33%)	28 (19%)	221 (36%)
NNPDS/Liquid faeces day one[2]	34/45	12/47	12/28	6/23	64/143

[1]NNPDS was defined as diarrhoea at any point during the second to fifth day of life.
[2]Proportion of piglets having liquid faecal consistency day one that developed NNPDS.

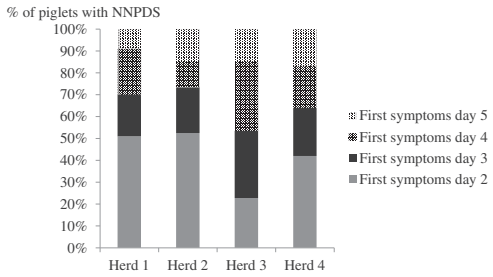


Figure 1 First day with symptoms in 160, 102, 88 and 69 NNPDS affected piglets in Herds 1, 2, 3 and 4.

consistency day one had similar effects in all herds; however, the effects were only statistically significant within Herd 4 and Herd 2, respectively. The odds of developing NNPDS were increased by 1.1 per 100 g decrease in birth weight (significant in Herd 4). Liquid consistency of faeces on the day of birth increased the odds of developing NNPDS 1.7 times (significant in Herd 2). Since treatment of sows was highly prevalent in Herd 4, a treatment variable (Yes/No) was also tested within this herd. Treatment of sows was not associated with the development of NNPDS (data not shown).

Discussion

The inclusion of herds for this study was based upon a high prevalence of unexplained neonatal diarrhoea prior to investigation, and intensive diagnostic investigations had suggested that they were all suffering from the emerging syndrome, NNPDS [2]. However, the low prevalence of diarrhoea during the study-period in Herd 4, suggested that this herd was in fact in remission at the point of the study (this was confirmed by follow-up interviews). Since many sows in this herd were medicated on the day of farrowing, the low prevalence of diarrhoea could be hypothetically linked with this. However, data did not support this theory and according to the herd-manager the rate of medication was not higher than in preceding periods with a high prevalence of diarrhoea.

According to interviews with herd-managers, the prevalence of diarrhoea during the study-periods in Herds 2 and 3 was slightly lower than normal, but otherwise reflected their normal situations quite well. A lower prevalence in the study-period can be explained by elements in the study-design made in order to evaluate risk-factors and avoid excess mortality. Thus, restricting litter sizes to a minimum, prohibiting cross-fostering as well as excluding underweight piglets could explain a lower prevalence of diarrhoea than normal. The severe symptoms in Herd 1 matched recordings carried out before and after the study-period. Investigated sow and piglet level risk factors did not give any obvious explanation for this herd to stand out. As previously published, pathological and microbiological findings in piglets from this herd did not differ markedly from findings in the remaining herds [2]. Furthermore, seasonal variations were unlikely to play a role, since Herd 1 was investigated during winter, which is the low season for neonatal diarrhoeas [3,7].

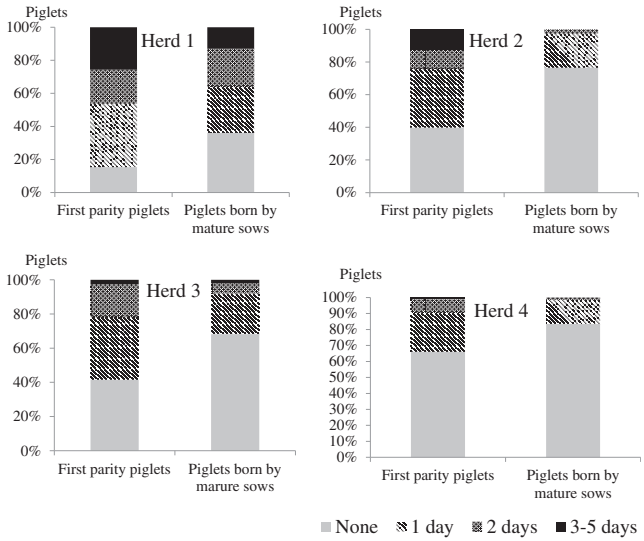


Figure 2 Duration of NNPDS. Legend: The figure shows the number of days that piglets were diarrhoeic between day two and five of life. Piglets that were euthanized or died prior to day five of life were not included in these data.

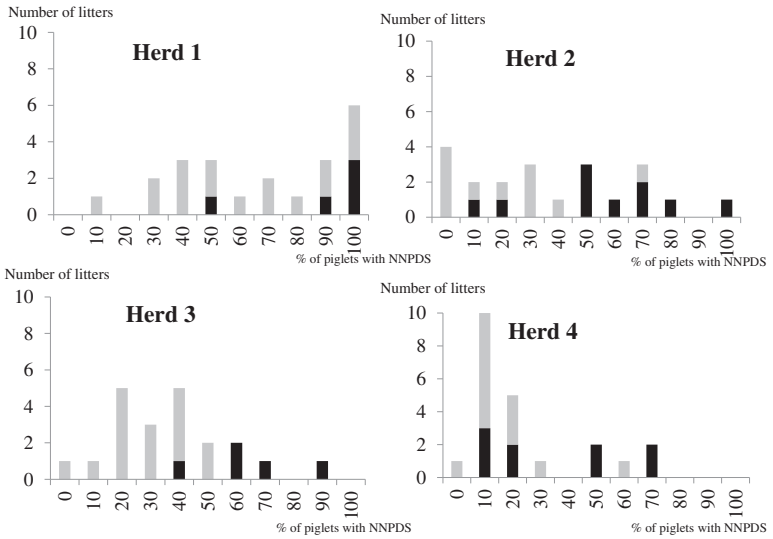


Figure 3 Prevalence of NNPDS within litters in the four herds. Legend: First parity litters are presented in black. A 10% prevalence equals approximately one piglet.

Table 5 Results of the overall generalized linear mixed model on NNPDS

Risk factor	Coefficient	SE	OR^1_{PA}	P-value
Intercept	−1.63			
Herd[2]				<0.001
Herd 4	0[a]			
Herd 2	0.49[a,b]	0.36	1.4	
Herd 3	1.11[b]	0.37	2.7	
Herd 1	2.68[c]	0.38	12.8	
Parity				< 0.001
2nd-7th	0			
1st	1.54	0.28	4.1	
Birth weight				0.003[3]
Per 100 g	−0.09	0.03	0.8	
Day one faecal consistency				0.006[3]
Normal	0			
Liquid	0.54	0.19	1.5	
ICC[4] litter	21%			

[1]The population average OR accounts for an average piglet in any litter.
[2]Herds with different letters as superscript are significantly different ($\alpha < 0.05$) when compared pairwise. [3]Note: This model did not include interaction effects – therefore these effects are finally evaluated in the herd specific models. [4]Intra Class Correlation Coefficient (the percentage of total variation in data that is explained by the random litter effect).
Risk factors evaluated included: Herd, parity of sow, number of stillborn, clinical disease in sow, gender, birth weight, faecal consistency, appearance of flanks and appearance of hair coat.

The study intended to describe the epidemiological pattern of NNPDS in terms of prevalence, timing, duration and tendency to cluster within litters. However, important limitations of the study that are relevant in the interpretation of its results need to be mentioned. An obvious limitation of the study was the fact that piglets were only examined for five days. Thus, the definition on NNPDS used in the study was made on practical grounds and should not be interpreted as if NNPDS does not occur beyond the fifth day of life. In fact, 25-50% of piglets within herds started having symptoms on the fourth or fifth day of life, and some of them were probably diarrhoeic beyond the period of examination.

In Herds 1 and 2, the risk of developing diarrhoea was 6–7 fold increased when born by a first parity sow. In the other herds, the association with parity was weaker, but still significant. An association with young sows is in line with previous studies on suckling piglet and neonatal diarrhoea [3,7,8]. Different factors, such as lower levels of colostral antibodies [9], differences in milk composition [10] and stressful behaviour in first parity sows [11] may explain this association. Although no specific microorganism has been identified in the pathogenesis of NNPDS the overrepresentation of first parity litters may be due to lack of specific colostral immunity of a yet unknown infectious agent. It seems intriguing to interpret a tendency to cluster within litters (as seen in Herd 1 and 2) as an indication of the syndrome being of infectious nature. However, inborn (genetic or developmental) and environmental factors are

Table 6 Results of herd-specific generalized linear mixed models on NNPDS

Risk factor	Herd 1				Herd 2				Herd 3				Herd 4			
	Coef.	SE	OR_{PA}[1]	*P*	Coef.	SE	OR_{PA}	*P*	Coef.	SE	OR_{PA}	*P*	Coef.	SE	OR_{PA}	*P*
Intercept	0.9				−1.9				−0.7				−1			
Parity				**0.03**				**<0.01**				**<0.01**				**0.04**
2nd-7th	0				0				0				0			
1st	2.1	0.99	**6.3**		2.2	0.6	**7.9**		1.4	0.3	**4**		1	0.5	**2.4**	
Birth weight				0.11				0.38				0.55				**0.02**
Per 100 g increase	−0.1	0.06			−0.1	0.07			−0.1	0.05			−0.2	0.06	**0.8**	
Faecal consistency				0.14				**0.04**				0.62				0.12
Normal	0				0				0				0			
Liquid	0.7	0.45			0.7	0.3	**1.7**		0.2	0.38			0.6	0.4		
ICC[2] litter	39%				26%				1%				15%			

[1]The population average OR accounts for an average piglet in any litter.
[2]Intraclass Correlation Coefficient (the percentage of total variation in data that is explained by the random litter effect).
Variables inserted in the full models included parity, birth weight and fecal consistency day one. OR's are only displayed for variables with significant effect. Estimates for non-significant variables were extracted from the un-reduced models. Significant associations are presented in bold.

also likely to cluster within litters, thus could also be a part of the explanation.

Obvious health problems in sows were rare and were not statistically associated with development of NNPDS. The lack of association between sow disease and NNPDS is interesting, since it might differentiate this syndrome from previously known neonatal diarrhoeas [3-5]. However, sows were only examined on the day of parturition and may have developed clinical symptoms later that were not taken into account. This study-design was chosen in order to be certain on cause-effect relationships (with piglets developing symptoms on different time-points, it seemed too difficult to evaluate the effect of clinical disease in sows during the whole study-period). In this study, the actual disease effects were probably integrated in the– highly variable – random effects of litters. Thus, the random litter effects probably represented a combination of undiagnosed disease, genetics and local environmental conditions as well as perhaps an infectious agent spreading within litters.

The fact that clinical signs of failure to thrive in piglets at day one were very infrequent (a total of 2% of piglets had protruding ribs) suggested that prenatal nutrition was generally adequate. Furthermore, since hollow flanks on the first day of life were not associated with the development of diarrhoea in these herds, symptoms seemed not to be caused by insufficient nutrition.

Potential associations between consistency of faeces on day one and the development of NNPDS were of major interest in this study, since a previous study suggested liquid faeces at birth to be a normal finding in these herds [6]. The present study showed that many piglets (46%) having liquid faeces at birth did not develop NNPDS, and that consistency of faeces at birth was only a minor risk factor for developing NNPDS. The decision to of the study to draw a sharp line between day one liquid faeces and day two NNPDS may be problematic, since so many piglets (20-50% of piglets within herds) were found to experience the first symptoms of NNPDS on day 2. Some of these piglets (perhaps especially within Herd 2) probably experienced the first symptoms of NNPDS on day one. The decision to draw this sharp line was made on practical grounds, in order to be able to evaluate the hypothesis of liquid faeces on the day of birth being unrelated to the syndrome. Since the study did (weakly) associate liquid faeces day one with the development of NNPDS, future studies should not rule out that NNPDS sometimes starts on the day of birth.

Naturally, the overall limitation of the study is the lacking definition on NNPDS. Thus, it is important to underline that the conclusions from this study may not apply to all cases of NNPDS, since they were drawn from findings in four herds only. However, the four herds were all thoroughly investigated in terms of possible infectious aetiologies, and none of them were diagnosed with any well-known agent to explain the symptoms. Therefore, it presently seems fair to consider the diarrhoeal outbreaks in these herds to represent NNPDS.

Conclusions

The prevalence and the duration of diarrhoea as well as the tendency of diarrhoea to cluster within litters differed much between herds diagnosed with NNPDS. In most cases, symptoms started on the second day of life, but in 25-50% of cases within herds symptoms started on the fourth or fifth day of life. The duration of diarrhoea was most often one to two days.

Herd of origin and sow-parity were the most important factors associated with the development of NNPDS, whereas birth weight and faecal consistency on the day of birth were less important risk factors. The study did not point out other sow-level risk factors than parity. The

reason for the more severe outbreak of NNPDS in Herd 1 was not explained by factors addressed in this study.

The general hypothesis of liquid faeces day one to be unrelated to the syndrome did not hold true. However, taking all results together, it seems that a liquid consistency faeces at birth is sometimes a harmless phenomenon, unrelated to NNPDS.

The study did not evaluate factors associated with the high prolificacy of Danish genetics (such as longer duration of farrowing) or herd-factors associated with NNPDS. Further studies are needed to look into these aspects.

Methods

Ethical approval

The study was conducted in accordance with the guidelines of the Danish Ministry of Justice with respect to animal experimentation and care of animals under study. According to Danish legislation this type of study does not require ethical approval.

Selection of herds

Four well-managed conventional herds were selected based on these criteria: 1) Persistent problems with diarrhoea during the first week of life with a poor response to antibiotic treatment, 2) Vaccination of sows against *Escherichia coli* and *Clostridium perfringens* type C, 3) Failure of preventive management interventions, 4) PRRS negative farrowing unit as demonstrated in blood samples tested by ELISA/IPT or PCR and 5) Negative results of routine diagnostic examination for enteritis in five piglets. All herds had a history of neonatal diarrhoea for a period of at least one year. Detailed interviews with herd owners, local veterinarians and feed consultants as well as preliminary herd visits were performed in order to exclude herds with obvious management related problems. Prior to the onset of investigations, herd-managers were instructed to carry out prevalence counts of diarrhoea in order to document a relatively high and constant prevalence of diarrhoea (rough estimates – data not shown). Furthermore, herds were instructed not to change any routines before, during or immediately after the study period. After the study periods, follow-up interviews were carried out in order to evaluate if the clinical picture had changed after leaving the herds. Herd 1, 2, 3 and 4 were investigated in January, March, May and July of 2011, respectively.

Diagnostic examinations on a total of 101 Case- and Control piglets from these herds ruled out that well-known infectious agents (enterotoxigenic *E. coli*, *Clostridium perfringens* type C, rotavirus A, coronavirus and *Cystoisospora suis*) could explain the aetiology of diarrhoea. Furthermore, previous studies suggested that neither *Clostridium perfringens* type A, *Clostridium difficile*, *Strongyloides ransomi*, *Giardia spp* nor *Cryptosporidium spp* was involved in the diarrhoeal outbreaks. Gross-pathologically, affected piglets were characterized by flaccidity of intestines with no mucosal lesions and milk-filled stomachs [2].

Study design and case-definition

The study was carried out as a cross-sectional study with follow-up during the first five days of piglets' lives in four herds previously diagnosed with NNPDS [2]. On each day of the study, rectal swabs were used to evaluate whether piglets were diarrhoeic (liquid or watery consistency of faeces) or not (creamy, firm and solid consistencies or absence of faeces on swab). NNPDS was defined as diarrhoea at some point during the second to fifth day of life (day one was hypothesized not to be part of the syndrome and piglets were only evaluated until the fifth day of life). Clinical explanatory variables in sows and piglets were registered on the day of parturition/birth (day one).

Inclusion of sows and piglets

In each herd, approximately 20 sows (6–8 per day) from one farrowing batch were selected on the day of parturition. All selected sows were situated in the same farrowing section. In herds predominantly experiencing problems in first parity litters (Herd 2 and 4), first parity sows were given high priority in the inclusion procedure. Otherwise, the sows that first finished farrowing on the major farrowing days were selected. At selection, litters were standardized to11 (Herd 1 and Herd 3) or 12 piglets (Herd 2 and Herd 4). Piglets to stay in the litters were selected by simple random sampling among littermates having a birth weight above 800 grams. Smaller piglets were excluded, since they were not expected to be able to survive among large litter-mates. All included piglets were kept in their original litters during the whole study period in order to be able to recognize sow-effects. During the study, selected piglets were euthanized for diagnostic purposes. Piglets euthanized without symptoms (Control piglets for the Case–Control study) were removed from data. Data used in the description of duration of symptoms only included piglets still present in the herds on day five of life.

Treatments during the study period

No preventive antibiotic medication of sows was given. Injection of oxytocin postpartum was accepted if recommended by the local veterinarian. Medical treatment of sows was carried out according to individual herd routines and involved antibiotics and NSAIDs. Decisions to treat were made by the herd-staff and not based on clinical registrations made in the study.

As a general rule, antibiotic treatment of piglets was not accepted within the first three days of life. Later in the study period, clinical diseases were treated according to individual herd routines. Non-antibiotic oral supplements to piglets were allowed during the whole study period.

Clinical examinations on day one

For each sow, parity, litter size and number of stillborn were registered. All sows were clinically examined between 5 and 20 hours postpartum. The clinical examination included assessment of udder, legs and vulva and registration of rectal temperature. Mastitis was recorded when one or more udder sections were firm, red or sore at palpation. Leg problems were recorded when sows were unwilling to bear equal weight on all legs or evaded palpation of legs or hooves. An excess of unclear or foul-smelling discharge from vulva was recorded as vulva discharge.

Piglets were weighed. The presence of hollow flanks, protruding ribs, rough hair coats, dehydration (lack of skin-elasticity and sunken eye-balls), skin-abrasions on fore-knees and faecal staining of perineum was dichotomously recorded. Faecal staining was assessed within a diameter of one cm around anus. By use of rectal swabs, consistency of faeces was evaluated as either normal (creamy, firm and solid consistencies or absence of faeces on swab) or liquid (liquid or watery consistencies).

All procedures in the herds were carried out by the same person.

Statistical analyses

Generalized linear mixed models with litter as random effect were used to evaluate potential risk factors for NNPDS. Due to a low prevalence of clinical disease in sows, all disease variables (mastitis, vulva discharge, leg problems and fever) were combined into one. An overview of all risk factors included in the analyses is given in Table 7.

Models were fit in R [12] using the lme4 package [13]. Model reduction was carried out using stepwise backwards elimination, removing variables with $p > 0.05$. Confounding was assessed by taking out and re-entering variables into the final models one by one and check for biologically important changes of estimates. Interaction terms were not included due to problems with complete separation, caused by sparse data. The linearity of birth weight at the log odds scale for NNPDS was assessed by transforming birth weight into a categorical variable based on quartiles and then verify that a decreasing trend of the estimates for the levels of the categorical variable was observed.

Table 7 Description of risk factors evaluated in the study

Risk-factors	Level	Categorization
Herd[1]	1,2,3,4	
Sow-related factors		
Parity	Young	1st parity
	Mature	2nd-7th parity
Litter size	Large[2]	Gilts: >15 piglets, Sows: >18 piglets
	Small	Gilts: <16 piglets, Sows: < 19 piglets
Stillborn	Many	>1 piglet
	Few	0-1 piglets
Clinical disease	Yes	Mastitis and/or temp > 39.5°C and/or leg problems and/or vulva discharge[3]
	No	None of the above
Piglet-related factors[4]		
Gender	Male	
	Female	
Birth weight	Continuous scale	
Faecal consistency	Liquid	Watery or liquid consistency of rectal contents
	Normal	Creamy, firm or solid consistency of rectal contents and if no faeces on swab
Flanks	Hollow	Area behind ribs turned inwards
	Normal	Area behind ribs followed the line of the ribs
Hair coat	Rough	Hair coat appeared dull
	Normal	Hair coat did not appear dull

[1]No herd effect was included in the overall parity-separated models.
[2]Litters above mean size of the parity group in question were considered large.
[3]Mastitis: One or more udder section hard, red or sore when palpated. Leg problems: The sow was unwilling to bear weight on all legs or sore at palpation. Vulva discharge: An excess of unclear or foul-smelling discharge.
[4]Protruding ribs, fore-knee abrasions and dehydration were too low prevalent to be included in the statistical models.
All clinical signs were assessed on the day of parturition/birth.

Population average Odds Ratios (OR_{PA}) were calculated using the following formula:

$$OR_{PA} = \exp(\beta_{SS})/\mathrm{sqrt}(1 + 0.346 * \delta^2_{litter})$$

where β_{SS} is the Subject Specific regression coefficient and δ^2_{litter} is the litter variance. The constant 0.346 is an approximation of the residual variance [14].

Population averaged, rather than cluster specific estimates of OR's were used, since the study aimed at drawing general rather than litter-specific conclusions.

Pairwise post-hoc comparisons within significant variables with more than two levels were carried out using the lsmeans package in R [15].

Since data was limited, modelling was performed using a three-step procedure in order to be able to identify all associations of practical interest.

Step 1: Two separate models – one for first parity piglets and one for piglets born by mature (2nd-7th parity) sows -were run to identify overall sow-related risk-factors for NNPDS. The separation into two models was done in order to be able to recognize possible differences in sow-effects in first parity vs. mature sows. Furthermore, since first parity sows had smaller litters, different cut-offs were needed in the evaluation of the effect of litter size. In each model, litter size was inserted dichotomously with cut-off at the mean litter size in question (15 and 18, respectively). Herds were not included in these models, in order to avoid overlooking any subtle sow-effects.

Step 2: If the results of the parity-specific models in step 2 were alike, an overall model including parity and herd-effects was generated.

Step 3: Separate models for each herd were used to evaluate risk factors and random litter effects within the separate herds. These models were furthermore used to properly evaluate piglet-level risk-factors (due to the previously mentioned inability to include interaction terms in Step 2).

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
All authors contributed to the design of the study. JPN contributed to the practical design of the field studies and NT contributed to decisions on the statistical approach of the study. Inclusion of herds, clinical examination in the herds and statistical analyses were performed by HK. All authors participated in drafting the manuscript and proofreading of the manuscript. All authors read and approved the final manuscript.

Acknowledgements
We would like to thank herd-owners and their staff for help and cooperation. Furthermore, we would like to thank Amalie Bækbo, Mikkel Haugegaard, Lars Jensen and Camilla Bjørn Olesen for practical assistance in the herds.

Author details
[1]Danish Pig Research Centre, Danish Agriculture and Food Council, Vinkelvej 13, 8620 Kjellerup, Denmark. [2]Department of Large Animal Sciences, HERD – Centre for Herd-oriented Education, Research and Development, University of Copenhagen, Groennegaardsvej 2,1870 Frederiksberg C, Denmark. [3]National Veterinary Institute, Technical University of Denmark, Bülowsvej 27, 1870 Frederiksberg C, Denmark.

Received: 1 March 2014 Accepted: 1 July 2014
Published: 10 July 2014

References
1. Kongsted H: *Spædgrisediarré i Danmark Anno 2013 (Danish).* http://vsp.lf.dk/~/media/Files/PDF%20-%20Publikationer/Erfaringer%202013/Erfaring_1320_Sp%C3%A6dgrisediarre_ i_Danmark_anno_2013.ashx.
2. Kongsted H, Jonach B, Haugegaard S, Angen O, Jorsal SE, Kokotovic B, Larsen LE, Jensen TK, Nielsen JP: **Microbiological, pathological and histological findings in four Danish pig herds affected by a new neonatal diarrhoea syndrome.** *BMC Vet Res* 2013, **9:**206.
3. Svensmark B, Jorsal SE, Nielsen K, Willeberg P: **Epidemiological studies of piglet diarrhoea in intensively managed Danish sow herds. I. Pre-weaning diarrhoea.** *Acta Vet Scand* 1989, **30**(1):43–53.
4. Wittum TE, Dewey CE, Hurd HS, Dargatz DA, Hill GW: **Herd- and litter-level factors associated with the incidence of diarrhea morbidity and mortality in piglets 1 to 3 days of age.** *Swine Health Prod* 1995, **3**(3):99–104.
5. Lingaas F: **Epidemiological and genetical studies in Norwegian pig herds. IV. Breed effects, recurrence of disease, and relationship between disease and some performance traits.** *Acta Vet Scand* 1991, **32**(1):107–114.
6. Kongsted H, Stege H, Toft N, Nielsen JP: **The effect of New Neonatal porcine Diarrhoea Syndrome on average daily gain and mortality in four Danish pig herds.** *BMC Vet Res* 2014, **10:**90.
7. Lingaas F, Ronningen K: **Epidemiological and genetical studies in Norwegian pig herds. II. Overall disease incidence and seasonal variation.** *Acta Vet Scand* 1991, **32**(1):89–96.
8. Sialelli J, Lautrou Y, Oswald I, Quiniou N: **Peut-on établir une relation entre les charactéristiques de la trui et de sa portée et l'apparition des diarrhées néonathales? [abstract].** *Journées Recherche Porcine* 2009, **41:**167–172.
9. Fairbrother JM, Gyles CL: **Colibacillosis.** In *Diseases of Swine.* 10th edition. Edited by Zimmerman JJ, Karriker LA, Ramirez A, Schwartz KJ, Stevenson GW. Iowa, USA: Wiley-Blackwell; 2012:723–749.
10. Beyer M, Jentsch W, Kuhla S, Wittenburg H, Kreienbring F, Scholze H, Rudolph PE, Metges CC: **Effects of dietary energy intake during gestation and lactation on milk yield and composition of first, second and fourth parity sows.** *Arch Anim Nutr* 2007, **61**(6):452–466.
11. Pedersen LJ, Jensen T: **Effects of late introduction of sows to two farrowing environments on the progress of farrowing and maternal behavior.** *J Anim Sci* 2008, **86**(10):2730–2737.
12. CoreTeam R: *R: A Language and Environment for Statistical Computing.* Vienna, Austria: R foundation for statistical computing; 2013. http://www.Rproject.org/.
13. Bates D, Maechler M, Bolker B: *lme4: Linear Mixed-Effects Models Using S4classes.* 2013. http://www.CRAN.R-project.org/package=lme4.
14. Dohoo I, Martin W, Stryhn H: *Veterinary Epidemiologic Research: 2nd ed.* Charlottetown, Prince Edward Island, Canada: VER Inc; 2009.
15. Lenth RV: *lsmeans: Least-Squares Means.* 2013. http://www.CRAN.R-project.org/package=lsmeans.

doi:10.1186/1746-6148-10-151
Cite this article as: Kongsted *et al.*: Risk factors and epidemiological characteristics of new neonatal porcine diarrhoea syndrome in four Danish herds. *BMC Veterinary Research* 2014 **10**:151.

5.4 Manuscript 4

No evidence of a viral involvement in the new neonatal porcine diarrhoea syndrome in Danish pigs

Larsen L.E.*, Hjulsager C.K., Boye M., Rasmussen S., Granberg F., Fischer T.K., Midgley S.E., Rasmussen L.D, Kongsted H., Angen Ø., Nielsen J.P.

*Corresponding author:

Lars Erik Larsen, National Veterinary Institute, Technical University of Denmark

Phone: +4535886274

Email: lael@vet.dtu.dk

No evidence of a viral involvement in the new neonatal porcine diarrhoea syndrome in Danish pigs

LE Larsen[1*], CK Hjulsager [1], M Boye[1], S Rasmussen[1], F Granberg[2], TK Fischer[3], SE Midgley[3], LD Rasmussen[1], H Kongsted [4,5], Ø Angen [2,6], JP Nielsen[5]

[1] National Veterinary Institute, Technical University of Denmark, Bülowsvej 27, 1870 Frederiksberg C, Denmark

[2] Department of Biomedical Sciences and Veterinary Public Health (BVF), Swedish University of Agricultural Sciences (SLU), Uppsala, Sweden

[3]Statens Serum Institut (SSI); Artillerivej 23 - DK-2300 Copenhagen S, Denmark

[4] Pig Research Centre, Danish Agriculture and Food Council, Vinkelvej 13, 8620 Kjellerup, Denmark

[5]HERD – Centre for Herd-oriented Education, Research and Development, Department of Large Animal Sciences, University of Copenhagen, Groennegaardsvej 2, 1870 Frederiksberg C, Denmark

[6]Present address: Norwegian Veterinary Institute, POBox 750 Sentrum, 0106 Oslo, Norway

* Corresponding author. Tel: +45 40998434

E-mail address: lael@vet.dtu.dk

Abstract

The aim of this study was to investigate if a virus is involved in the syndrome New Neonatal Porcine Diarrhoea Syndrome (NNPDS). Four well-managed herds experiencing neonatal diarrhoea and suspected to be affected by NNPDS were selected, and 989 piglets within these herds were clinically examined on a daily basis. Samples from diarrhoeic and non-diarrhoeic piglets at the age of three to seven days were selected for extensive virological examination using specific PCRs and general virus detection methods.

Using specific PCRs, a total of 9% of tested animals were rotavirus A positive. No animals tested positive for rotavirus C, coronavirus (TGEV/ PEDV), Astrovirus, Sapovirus, Enterovirus, Parechovirus, Saffoldvirus, Cosavirus, Aichivirus or Klassevirus. Microarray-analyses performed on a total of 18 animals were all negative, as were eight animals examined by Transmission Electron Microscopy. Using Next Generation *de novo* sequencing on pools of samples from case animals within all herds, kobuvirus, rotavirus A, rotavirus C and teschovirus were detected in 4,1,1 and 1 herds, respectively.

It was concluded that the analyses performed on enteric samples in the study did not sustain a significant contribution of viruses to NNPDS. Still, a systemic virus may play a role in the pathogenesis of NNPDS and further investigations are needed to scrutinize that possibility.

Keywords

Diarrhoea, neonatal piglets, NNPDS, virus

Background

Since 2008, field experiences on a new diarrhoeic syndrome in neonatal piglets referred to as New Neonatal Porcine Diarrhoea Syndrome (NNPDS) has been reported in Denmark and elsewhere (Gin et al., 2010; Melin et al., 2010; Svensmark, 2009; Wallgren et al., 2012). The prevalence of well-known enteric pathogens, gross- and histological findings in age-matched diarrhoeic- and non-diarrhoeic piglets from four Danish herds have previously been reported (Kongsted et al., 2013). Briefly, no association between the presence of diarrhoea and the detection of enterotoxigenic *Eherichica. coli, Clostridium perfringens* type A or C, Rotavirus A, Coronavirus, *Clostridium difficile*, *Cryptosporidium spp*, *Giardia spp*, *Cystoisospora suis* or *Strongyloides ransomi* was revealed. The conclusion of these detailed examinations was that no known single causative pathogen could be related to the presence of neither clinical disease nor pathological lesions (Jonach et al., 2014; Kongsted et al., 2013).

The aim of the present study was to complete a more detailed investigation on possible viral involvement in NNPDS. Selected samples from the previously examined herds were examined for the presence of a range of specific viruses previously related to enteric conditions in pigs and other species. In addition, selected samples were investigated for the presence of virus in general by Transmission Electron Microscopy, pan-viral microarray and *de novo* sequencing using next generation sequencing (NGS).

Materials and Methods

Herds and animals

Four well-managed herds affected by severe neonatal diarrhoea for at least one year were selected for the study. Approximately 15 Case (diarrhoeic) and 15 Control (non-diarrhoeic) piglets per herd were selected for euthanasia. All selected piglets were at the age of three to seven days of age. In addition to euthanized animals, a selected number of rectal swabs from the day of euthanasia were included in the study. For details on inclusion criteria and definitions on Case and Control animals see Kongsted et al. (2013).

Selection and storing of tissue samples

Live piglets were transported to the laboratory and euthanized within six hours after selection in the herds. Immediately after euthanasia samples from ileum were snap-frozen on dry ice and stored at -80 °C until further use. Rectal swabs taken in the herds were frozen immediately and kept in a freezer with dry-ice in the herds until transportation to the laboratory, where they were stored at -80°C until further use.

Extraction of RNA for Corona and Astrovirus RT-PCR analysis

Up to 40 mg ileum with content was homogenized in 300 µl chilled 1-Thioglycerol Homogenization Solution (Promega) on a TissueLyserII (QIAGEN) at 20 Hz for 2 min, vortexed and heated at 70°C for 2 min and then stored on ice. 300 µl of lysisbuffer were mixed into to the sample and 10 µl DNase added afterwards. RNA was extracted from all of the homogenate on a Maxwell® automated purification robot with the Maxwell® 16 LEV SimplyRNA Tissue Kit (Promega) according to instructions from the supplier and eluted in 70 µl nucleic free water.

Coronavirus RT-PCRs

The samples were initially tested by a conventional pan-corona RT-PCR assay which has been designed to detect a wide range of coronaviruses (Vijgen et al., 2008).

Subsequently in house real time RT-PCR assays were used to test samples specifically for Porcine Epidemic Diarrhoea virus (PEDV), porcine respiratory corona virus (PRCV) and transmissible gastroenteritis virus (TGE). TGE and PRC were detected simultaneously in a duplex realtime RT-PCR by combining two published assays (Kim et al., 2007; Vemulapalli et al., 2009). The real time RT-PCR was performed as a one-step RT-PCR reaction using RNA UltraSense ™ One-Step Quantitative RT-PCR System (Invitrogen). Primer and probe concentrations and the PCR assay conditions were as described using the MX3005p qPCR system (Stratagene). One minor modification was made since the TGE probe had the fluorescent dye Cy5 at the 5' end to distinguish its signal from the FAM marked TGE/PRC probe. Detection of PED was performed under the conditions described by Kim et al. (2007) with the modifications that the real time RT-PCR was performed as a one-step RT-PCR reaction using RNA UltraSense ™ One-Step Quantitative RT-PCR System (Invitrogen) and the reaction was run on the MX3005p qPCR system (Stratagene).

Astrovirus RT PCR

A real time RT-PCR assay was established to test the samples for astrovirus. Up to 40 mg ileum with content was homogenized in 300 µl chilled 1-Thioglycerol Homogenization Solution (Promega) on a TissueLyserII (QIAGEN) at 20 Hz for 2 min, vortexed and heated at 70°C for 2 min. RNA was extracted from 300 µl homogenate on a Maxwell® automated purification robot with the Maxwell® 16 LEV SimplyRNA Tissue Kit (Promega) according to instructions from the supplier. Real-time RT-PCR was performed in a total volume of 15 µl containing 2 µl RNA, 400 nM primer MiAstV-F 5'-TCTTRATGCYCAGTGGTGAGGT-3', 400 nM primer MiAstV-R 5'-CTGGAAGAACACGTTGCACAAAT-3', 133 nM 6-FAM MGBNFQ MiAstVprobe with the sequence 5'-ACCCGYCAGACCAAGGGCAAYCCA (Applied Biosystems), 1xRT-PCR Buffer and 1xRT-PCR Enzyme Mix (AgPath-ID™ One-Step RT-PCR Kit, Life Technologies). PCR cycling was carried out on RotorGene3000 or RotorGeneQ (QIAGEN) with the following profile: 45°C for 10 min., 95°C for 10 min., 48 cycles 95°C for 15 sec, 60°C for 45 sec. The fluorescence signal was acquired at the 60°C step in the Green channel (470 nm). Data was analyzed with the RotorGene software (QIAGEN) in the green channel using dynamic tube normalization, noise slope correction and 10% no template control threshold. Samples were run in duplicate in parallel with positive and negative controls. Reactions were evaluated positive if the normalized fluorescent signal reached a threshold set at 0.02. The procedure was tested for inhibition by spiking of Mink astrovirus positive faeces into 5 randomly selected samples prior to RNA extraction and comparison by real-time RT-PCR to similarly spiked water.

Rotavirus (types A and C), Norovirus and Sapovirus RT PCRs

Contents of jejunum from all samples collected in the study were examined for rotavirus group A by an enzyme immunoassay (ProSpectT® Rotavirus) according to the manufacturer's instructions (Kongsted et al. 2013). The presence of noro- and sapoviruses was tested using a previously described multiplex real time PCR for NoV (GI & GII) (Kageyama et al., 2003) and Sapovirus (Hansman et al., 2007).

In the present study, real time assays were employed to test the samples for rotavirus A.

The real-time assay specific for rotavirus A virus targeting the NSP3 gene was designed to detect all rotavirus A viruses from humans and animals. Samples of ileum with contents were prepared as a 10% (weight/volume) suspension in minimal essential medium and centrifuged at 3500×g for 30 min. Nucleic acids were extracted from 200 µl sample material using the MagNa Pure LC Total Nucleic Acid Isolation Kit on the MagNaPure LC or MagNa Pure 96 (Roche Diagnostics) instruments according to the manufacturer's specifications. Real-time PCR was carried out using a previously published assay (Pang et al., 2004). Another real-time RT-PCR assay was used to test the samples for rotavirus C virus by targeting the VP7 gene out using a previously published assay (Logan et al., 2006). Samples were prepared as described for rotavirus A.
The samples were tested for sapovirus by a previously described real time RT-PCR assay (Oka et al., 2006) using the same sample preparation protocol and extraction procedures.

Enterovirus, parechovirus, saffoldvirus,cosavirus, aichivirus and klassevirus

Previously described real time RT-PCR assays were used to test for Enterovirus, Parechovirus, Saffoldvirus, Cosavirus, Aichivirus and Klassevirus (Nielsen et al., 2013a; Nielsen et al., 2013b) using the same sample preparation protocol as for rotavirus and Sapovirus.

General, unspecific virus analyses

Transmission Electron Microscopy (TEM)

The TEM analyses were performed on frozen ileum including content from animals with defined and severe villus atrophy (Jonach et al., 2014) by a commercial provider (Bio-imaging unit at Animal Health and Veterinary Laboratory (AH-VLA), Weybridge, UK) using standard methods at a magnification of 34000x. Confirmation of the presence of virus in a sample was based on size, shape, fine structure and surface morphological differentiation.

Microarray

The microarray analyses were performed on frozen ileum, including content from selected animals with defined and severe villus atrophy (Jonach et al., 2014). The microarray used consisted of a pan-viral microarray containing 47,000 probes covering all the virus entries in GenBank developed in cooperation with AH-VLA in the UK. The protocol for sample preparation and test of samples were as described by Gurrala et al., 2009. The estimated sensitivity of the assay is 3-6 log10 copies/sample.

Next Generation de novo sequencing (de novo NGS)

The NGS analyses were performed on frozen ileum including content from selected animals with defined and severe villus atrophy (Jonach et al., 2014). The samples were tested in pools of five animals from each of the four herds. Sample preparation and nucleic acid isolation was performed as previously described (Granberg et al., 2013). By making two parallel extractions of RNA and DNA from each sample, generating cDNA, and pooling the material from all samples in each crew, enough material was generated to avoid pre-amplification. Each pooled sample was sequenced on an Ion Torrent PGM system using the 200-bp read chemistry and an Ion 316 chip. This was performed as described earlier (Belak et al., 2013) at the Uppsala Genome Center, SciLifeLab, Sweden. The resulting reads were assembled using MIRA (Chevreux et al., 2004) with the standard settings for *de novo* assembly of Ion Torrent data. Taxonomic classification of assembled contigs was enabled by Blastn and Blastx searches against local copies of NCBI's nucleotide and protein databases using the Blast+ package (Camacho et al., 2009) with default settings. Evaluating the taxonomic data for potential viruses, candidate reference genomes were identified and retrieved from GenBank in FASTA format. Alignments of contigs against the nucleotide sequences of the reference genomes were performed using the CodonCode Aligner software (CodonCode Corporation).

Results

Coronaviruses

Samples from a total of 46 case animals and 46 control animals were tested for coronavirus (Table 1). Ileum tissue, ileum contents and rectal swabs from animals in herds 1, 2 and 3 and rectal swabs from animals in herd 4 were tested in the conventional pan-corona assay. All samples tested negative. Some of the samples generated a band at the correct size at the gel but subsequent sequencing revealed that the band represented unspecific amplification of porcine DNA (data not shown). Samples from all animals in herds 1-3 (Table 1) were tested in real time RT-PCR specific for PEDV, PRCV and TGEV with clear negative results.

Rotavirus

By ELISA, only one (case) animal tested positive for Rotavirus A. Using real time PCR, test of 76 samples from herds 1-3 (Table 1) revealed a total of 7 positive samples, of which 6 samples were

collected from case animals and 1 from a control animal. The numbers of positive samples in each herd were 2, 3 and 2 for herds 1, 2 and 3, respectively. The sample that tested positive for rotavirus in ELISA was the most positive sample in the real time PCR assay (cq value of 19).

All 76 samples tested for rotavirus C by real time RT-PCR yielded negative results.

Sapovirus and norovirus

Fecal swab samples from the 76 animals in herds 1-3 (table 1) were tested in real time RT-PCR specific for sapovirus and norovirus and gave clear negative results.

Astrovirus

No inhibition was observed for the astrovirus protocol based on Ct-values from spiked samples being 0.72 - 1.65 Ct-values lower than equally spiked water. Fecal swab samples from the 76 animals in herds 1-3 (Table 1) tested negative in real time RT-PCR specific for astrovirus.

Enterovirus, Parechovirus, Saffoldvirus, Cosavirus, Aichivirus and Klassevirus

A total of 13 rectal swab samples from case animals representing all 4 herds were tested for in real time assay specific for enterovirus, parechovirus, saffoldvirus, cosavirus, aichivirus and klassevirus. All samples yielded negative results.

Unspecific virus tests

Transmission Electron microscopy

Eight samples (ileum with content) from case animals with histological lesions typical of viral infections (villus atrophy) representing all 4 herds were analysed by TEM with negative results.

Microarray

A total of 18 samples from 13 case and 5 control animals were tested by microarray. None of the samples tested conclusive positive for any of the virus present on the pan-viral microarray

De Novo NGS

Samples from five case animals from each herd were pooled and tested by NGS. Endogenous retrovirus were present in all four pools (data not shown). Kobuvirus was also present in all four

pools as evident by several reads (Table 2). In addition, the pools of samples from herd 1 were positive for rotavirus C virus, herd 2 were positive for rotavirus A and herd 3 were positive for teschovirus. One of the samples included in the pool from herd 2 was the animal that also tested positive for rotavirus A by real time PCR and by ELISA.

Discussion

The present study is part of a series of studies focused on scrutinizing the cause of NNPDS in Danish pigs, which previously was shown not to be associated with known bacterial or parasitic pathogens (Jonach et al., 2014; Kongsted et al., 2013). Unlike respiratory diseases, very few viruses have been linked to diarrhea in Danish pigs and therefore the samples were initially tested only for rotavirus A virus and in a pan-coronavirus assay. A few animals were positive for rotavirus A and none tested positive for coronavirus in a conventional pan-coronavirus conventional RT-PCR.

The objective of the present study was to broaden the test for viruses by testing selected samples for a range of specific viruses described to be linked to enteric disorders in pigs or humans and to test for virus in general by the use of pan viral methods including metagenomic approaches.

During the last three decades, PCR has revolutionized viral diagnostics by increasing both the diagnostic sensitivity and specificity of the applied tests (Elnifro et al., 2000). The major drawback of PCR is that the design of the tests requires detailed knowledge of the viral sequence and that even small changes in the viral genome may lead to false negative results. Thus, PCR is not an effective tool to detect new, drifting or reemerging viruses. Classical virus cultivation is another diagnostic approach but is restricted to identify viruses that can be propagated in the specific cell culture systems used which a great proportion of viruses are not. During recent years newer techniques using a metagenomic approach such as microarrays and *de novo* sequencing have been developed and used to detect emerging and reemerging viruses in humans and in animals (Belak et al., 2013; Chen et al., 2011). The advantages of these techniques are that they can detect all viruses irrespectively of prior information on the virus genetic sequence. The only request is that the new virus has some level of identity to previously sequenced viruses in order to be picked up by the downstream bioinformatic filtering of the data.

Overall, the application of PCR on the samples of the present study resulted in very few positive results. The initial test of the samples for Rotavirus A virus by a commercial available ELISA generated only one positive sample (Kongsted et al., 2013). A total of seven samples tested positive

when all samples were retested in a real time RT-PCR assay specific for Rotavirus A. The difference between the outcome of tests by ELISA and RT-PCR probably reflects the higher sensitivity of the PCR which is also underlined by the fact that the ELISA positive sample was the sample with the lowest ct value (19) in the PCR. The relative low number of positives strongly indicated that rotavirus A was not a significant problem in these herds. Group C rotavirus were first identified in swine in 1980 (Saif et al., 1980). Diarrheal outbreaks associated with rotavirus have been documented in nursing, weaning and post-weaning pigs either alone or in mixed infection with other enteric pathogens (Saif and Jiang, 1994). In a recent study in the USA, a total of 380 contemporary and archived samples were tested and 19.5% found positive. Of the 128 samples collected in 2012, 23.5% from nursing piglets (aged <3 weeks) and 8.5% from weaned piglets were RVC positive, with a higher RVC frequency in diarrheic (28.4%) than in non-diarrheic (6.6%) piglets (Amimo et al., 2013). In the present study, none of the samples from pigs below one week of age were positive for Rotavirus C by PCR but the pool of samples from the pigs in herd 1 generated a signal for Rotavirus C with 26 matching reads. The differences in the outcome of the two tests is not clear since the PCR should be more sensitive than the NGS but it may be because that two different extractions from the same sample were used in the two tests. No data on RVC are available from pigs in Denmark so it is unclear if the negative results in these four herds reflected that the virus are not prevalent among Danish pigs or that the animals get infected at an later age, but the results indicate that this virus did not contribute to diarrhea in the investigated herds.

Test of samples specific for the two important porcine coronaviruses TGE and PED expectably generated negative results. These viruses have not been diagnosed in Denmark for decades, but PED has recently reemerged as a significant pathogen in Asia and North America (Huang et al., 2013).

The test of samples for sapovirus gave negative results indicating that sapovirus did not contribute to the diarrhoeic symptoms in the examined herds. Porcine sapovirus has experimentally been shown to induce mild to moderate diarrhoea in pigs (Flynn et al., 1988; Guo et al., 2001), but epidemiologic evidence for a causative role of sapovirus in natural cases of suckling pig diarrhoea is scarce. In a cross-national survey from 2010, sapovirus was detected in 3.3%, 13.3% and 35% of suckling pigs from Hungary, Spain and Slovenia, respectively, but there was no association between diarrhoea and detection of sapovirus (Reuter et al., 2010). In the same study, faecal samples from 57 2-8 weeks old pigs with diarrhoea from 31 Danish herds were tested for sapovirus revealing

positive results in 68% of the herds covering 44% of the pigs. The test used in the two studies were identical (Oka et al., 2006), but the difference in test results can be explained by the fact that only pigs less than one week of age were included in the present study.

Norovirus was not detected in any of the animals. Norovirus has been shown to be prevalent in slaughter pigs in USA (Scheuer et al., 2013) and in Belgium (Mauroy et al., 2008) whereas samples from pigs aged 1-16 weeks tested negative in Spain (Halaihel et al., 2010). In 2007, a screening of 56 samples received for diagnostic purposes from 31 Danish swine herds revealed that 16% of the herds were positive (own unpublished results). The age of the pigs included in the screening were not known but the result indicate that porcine norovirus is indeed prevalent in Danish herds and the negative outcome of the test described in the present study can be explained by the age of the tested animals

Also test of the samples for astrovirus gave negative results. Recent studies in USA have revealed that astrovirus can be detected in up to 64% of pigs with or without diarrhea (Mor et al., 2012; Xiao et al., 2013). In the suckling pigs aged 1-20 days the prevalence was only 26% compared to 75% in the nusery pigs indicating a protective effect of maternal antibodies and/or an extended incubation period (Xiao et al., 2013). As for sapovirus, the very young age of piglets in this study may explain the negative outcome of the PCR tests. Nevertheless, if astrovirus played a significant role for the development of diarrhea in the examined herds, positive outcome of tests of case animals would be expected.

Thirteen of the samples from case pigs were screened in a multiplex real time PCR developed to test samples from humans for the suspected emerging human enteric pathogens enterovirus, parechovirus, saffoldvirus, cosavirus, aichivirus (human kobuvirus) and klassevirus. As expected, these samples were negative and since there has been no reports on these virus being present in pigs it was decided to omit testing of the remaining samples.

The general virological analyses employed was so expensive that it was outside the projects economical funding to test all samples. The samples selected for these analyses were selected based on presence of profound villus atrophy as revealed by the detailed histopathological examinations described elsewhere (Jonach et al., 2014). Villus atrophy is a typical pathological lesion induced by viruses.

The pan viral microarray chip used in the present study included more than 40.000 probes covering all the viral enteritis present in the Genbank (Gurrala et al., 2009). The array failed to detect any virus in the relatively few (n=18) samples tested indicating that no or small amounts of virus particles was present. The detection level of the chip is relatively low compared to real time PCR and de novo sequencing (Frey et al., 2014). In accordance, eight of eight samples examined by TEM which also has a relatively lov level of detection also gave clear negative results.

Compared to other studies on pig feces, very few viruses were detected by *de novo* sequencing in the present study. A previous study conducted in a US farm detected kobuvirus in 23% of all reads and techovirus in 0.03% of the reads (Shan et al., 2011). The former study also detected astrovirus (22%) and enterovirus (14%) which was not detected in our study. Interestingly, they found that the prevalence of most viruses was not greater in animals with diarrhea than in healthy animals. With respect to kobuvirus an opposite relation was seen since 12 times more reads were positive for the healthy animals. The differences between the studies may be explained by difference in age of the animals tested but the most likely explanation is that the US study used random PCR amplification prior to library building which greatly improves the sensitivity but also introduces a bias, which can prevent a meaningful quantitative analysis (Karlsson et al., 2013).

In the present study, kobuvirus was detected in samples from all herds by *de novo* sequencing. A study conducted in 40 Korean herds detected a higher prevalence of Porcine kobuvirus in diarrhoeic vs. non-diarrhoeic piglets (Park et al., 2010). However, only 3 of 71 samples (4%) from diarrhoeic pigs were not co-infected with other pathogens. This finding indicates that kobuvirus probably is not a significant primary pathogen in natural cases of diarrhoea. A recent study from USA supports this conclusion (Verma et al., 2013). In this study, 25 of 114 samples (21.9%) from diarrhoeic pigs and 10 of 46 (21.7%) samples from healthy pigs were positive. However, none of these studies specifically evaluated the prevalence of kobuvirus in piglets within the first week of life.

Porcine teschoviruses (PTVs) belong to the genus teschovirus within the family Picornaviridae. Hitherto, PTVs have had 13 serotypes associated with a variety of clinical diseases. PTV-1 strains were associated with highly fatal, nonsuppurative encephalomyelitis of pigs (Teschen disease) in the 1930-1950s. Today, less virulent talfan strains of PTV-1 are more widespread, and PTVs are detected in swine herds worldwide often together with a variety of common swine pathogens (multi-infection status). In a Chinese study, PTV was found in 96.7% of 30 culled 4-8 weeks old post-weanling piglets by nested RT-PCR. They found that only non-suppurative encephalitis may

be marginal significantly attributed to infection by PTVs (Chiu et al., 2012) but other PTV types may cause other disease symptoms (Feng et al., 2007).

In order to clarify the role of teschovirus and kobuvirus in NNPDS, detailed quantitative PCR testing on a larger fraction of case and control animals needs to be performed. Interestingly, new variants of astrovirus in addition to kobuvirus, calicivirus and rotavirus A were detected in two individual samples from Hungarian suckling piglets with NNPDS like symptoms using the same metagenomic approach as used in the present study (Belak et al., 2013). Thus, tests for these specific variants of astrovirus may also be rewarding in Danish cases of NNPDS. In conclusions, the analyses performed on samples from four Danish herds in this study did not sustain a significant contribution of viruses to NNPDS. However, since only enteric samples were analysed further studies are needed to investigate if a systemic virus infection may play a major or contributing role in the pathogenesis of NNPDS.

References

Amimo, J.O., Vlasova, A.N., Saif, L.J., 2013. Prevalence and genetic heterogeneity of porcine group C rotaviruses in nursing and weaned piglets in Ohio, USA and identification of a potential new VP4 genotype. Veterinary microbiology 164, 27-38.

Belak, S., Karlsson, O.E., Blomstrom, A.L., Berg, M., Granberg, F., 2013. New viruses in veterinary medicine, detected by metagenomic approaches. Veterinary microbiology 165, 95-101.

Camacho, C., Coulouris, G., Avagyan, V., Ma, N., Papadopoulos, J., Bealer, K., Madden, T.L., 2009. BLAST+: architecture and applications. BMC Bioinformatics 10, 421.

Chen, E.C., Miller, S.A., DeRisi, J.L., Chiu, C.Y., 2011. Using a pan-viral microarray assay (Virochip) to screen clinical samples for viral pathogens. Journal of Visualized Experiments 50.

Chevreux, B., Pfisterer, T., Drescher, B., Driesel, A.J., Muller, W.E., Wetter, T., Suhai, S., 2004. Using the miraEST assembler for reliable and automated mRNA transcript assembly and SNP detection in sequenced ESTs. Genome research 14, 1147-1159.

Chiu, S.C., Hu, S.C., Chang, C.C., Chang, C.Y., Huang, C.C., Pang, V.F., Wang, F.I., 2012. The role of porcine teschovirus in causing diseases in endemically infected pigs. Veterinary microbiology 161, 88-95.

Elnifro, E.M., Ashshi, A.M., Cooper, R.J., Klapper, P.E., 2000. Multiplex PCR: optimization and application in diagnostic virology. Clinical microbiology reviews 13, 559-570.

Feng, L., Shi, H.Y., Liu, S.W., Wu, B.P., Chen, J.F., Sun, D.B., Tong, Y.E., Fu, M.S., Wang, Y.F., Tong, G.Z., 2007. Isolation and molecular characterization of a porcine teschovirus 1 isolate from China. Acta Virologica 51, 7-11.

Flynn, W.T., Saif, L.J., Moorhead, P.D., 1988. Pathogenesis of porcine enteric calicivirus-like virus in four-day-old gnotobiotic pigs. American Journal of Veterinary Research 49, 819-825.

Frey, K.G., Herrera-Galeano, J.E., Redden, C.L., Luu, T.V., Servetas, S.L., Mateczun, A.J., Mokashi, V.P., Bishop-Lilly, K.A., 2014. Comparison of three next-generation sequencing platforms for metagenomic sequencing and identification of pathogens in blood. BMC Genomics 15, 96.

Gin, T., Guennec, J.L., Morvan, H., Martineau, G.P., 2010. Clinical and laboratory investigations in 10 French pig herds dealing with enzootic neonatal diarrhea. In: Proceedings of the 21st IPVS Congres, Vancouver, Canada, pp. 758.

Granberg, F., VicenteRubiano, M., RubioGuerri, C., Karlsson, O.E., Kukielka, D., Belak, S., SanchezVizcaino, J.M., 2013. Metagenomic detection of viral pathogens in Spanish honeybees: co-infection by aphid lethal paralysis, Israel acute paralysis and Lake Sinai viruses. PLoS ONE 8:2, e57459.

Guo, M., Hayes, J., Cho, K.O., Parwani, A.V., Lucas, L.M., Saif, L.J., 2001. Comparative pathogenesis of tissue culture-adapted and wild-type Cowden porcine enteric calicivirus (PEC) in gnotobiotic pigs and induction of diarrhea by intravenous inoculation of wild-type PEC. Journal of Virology 75, 9239-9251.

Gurrala, R., Dastjerdi, A., Johnson, N., NunezGarcia, J., Grierson, S., Steinbach, F., Banks, M., 2009. Development of a DNA microarray for simultaneous detection and genotyping of lyssaviruses. Virus research 144, 202-208.

Halaihel, N., Masia, R.M., FernandezJimenez, M., Ribes, J.M., Montava, R., Blas, I.d., Girones, O., Alonso, J.L., Buesa, J., 2010. Enteric calicivirus and rotavirus infections in domestic pigs. Epidemiology and Infection; 2010.138: 4, 542-548.30 ref .

Hansman, G.S., Ishida, S., Yoshizumi, S., Miyoshi, M., Ikeda, T., Oka, T., Takeda, N., 2007. Recombinant sapovirus gastroenteritis, Japan. Emerging Infectious Diseases 13, 786-788.

Huang, Y.W., Dickerman, A.W., Pineyro, P., Li, L., Fang, L., Kiehne, R., Opriessnig, T., Meng, X.J., 2013. Origin, evolution, and genotyping of emergent porcine epidemic diarrhea virus strains in the United States. mBio 4, e00737-13.

Jonach, B., Boye, M., Stockmarr, A., Jensen, T., 2014. Fluorescence in situ hybridization investigation of potentially pathogenic bacteria involved in neonatal porcine diarrhea. BMC Veterinary Research 10, 68.

Kageyama, T., Kojima, S., Shinohara, M., Uchida, K., Fukushi, S., Hoshino, F.B., Takeda, N., Katayama, K., 2003. Broadly reactive and highly sensitive assay for Norwalk-like viruses based on real-time quantitative reverse transcription-PCR. Journal of clinical microbiology 41, 1548-1557.

Karlsson, O.E., Belak, S., Granberg, F., 2013. The effect of preprocessing by sequence-independent, single-primer amplification (SISPA) on metagenomic detection of viruses. Biosecurity & Bioterrorism 11, 227-234.

Kim, S., Kim, I., Pyo, H., Tark, D., Song, J., Hyun, B., 2007. Multiplex real-time RT-PCR for the simultaneous detection and quantification of transmissible gastroenteritis virus and porcine epidemic diarrhea virus. Journal of Virological Methods 146, 172-177.

Kongsted, H., Jonach, B., Haugegaard, S., Angen, O., Jorsal, S.E., Kokotovic, B., Larsen, L.E., Jensen, T.K., Nielsen, J.P., 2013. Microbiological, pathological and histological findings in four Danish pig herds affected by a new neonatal diarrhoea syndrome. BMC Veterinary Research 9, 206.

Logan, C., O'Leary, J.J., O'Sullivan, N., 2006. Real-time reverse transcription-PCR for detection of rotavirus and adenovirus as causative agents of acute viral gastroenteritis in children. Journal of clinical microbiology 44, 3189-3195.

Mauroy, A., Scipioni, A., Mathijs, E., Miry, C., Ziant, D., Thys, C., Thiry, E., 2008. Noroviruses and sapoviruses in pigs in Belgium. Archives of Virology 153, 1927-1931.

Melin, L., Wallgren, P., Mattsson, S., Stampe, M., Lofstedt, M., 2010. Neonatal diarrhoea in piglets from E.coli vaccinated sows in Sweden. In: Proceedings of the 21st IPVS Congress, Vancouver, Canada, pp. 290.

Mor, S.K., Chander, Y., Marthaler, D., Patnayak, D.P., Goyal, S.M., 2012. Detection and molecular characterization of Porcine astrovirus strains associated with swine diarrhea. Journal of Veterinary Diagnostic Investigation 24, 1064-1067.

Nielsen, A.C., Bottiger, B., Midgley, S.E., Nielsen, L.P., 2013a. A novel enterovirus and parechovirus multiplex one-step real-time PCR-validation and clinical experience. Journal of virological methods 193, 359-363.

Nielsen, A.C., Gyhrs, M.L., Nielsen, L.P., Pedersen, C., Bottiger, B., 2013b. Gastroenteritis and the novel picornaviruses aichi virus, cosavirus, saffold virus, and salivirus in young children. Journal of Clinical Virology 57, 239-242.

Oka, T., Katayama, K., Hansman, G.S., Kageyama, T., Ogawa, S., Wu, F.T., White, P.A., Takeda, N., 2006. Detection of human sapovirus by real-time reverse transcription-polymerase chain reaction. Journal of medical virology 78, 1347-1353.

Pang, X.L., Lee, B., Boroumand, N., Leblanc, B., Preiksaitis, J.K., Yu Ip, C.C., 2004. Increased detection of rotavirus using a real time reverse transcription-polymerase chain reaction (RT-PCR) assay in stool specimens from children with diarrhea. Journal of medical virology 72, 496-501.

Park, S., Kim, H., Moon, H., Song, D., Rho, S., Han, J., Nguyen, V., Park, B., 2010. Molecular detection of porcine kobuviruses in pigs in Korea and their association with diarrhea. Archives of Virology 155, 1803-1811.

Reuter, G., ZimsekMijovski, J., PoljsakPrijatelj, M., Bartolo, I.d., Ruggeri, F.M., Kantala, T., Maunula, L., Kiss, I., Kecskemeti, S., Halaihel, N. and others, 2010. Incidence, diversity, and molecular epidemiology of sapoviruses in swine across Europe. Journal of Clinical Microbiology 48, 363-368.

Saif, L.J., Bohl, E.H., Theil, K.W., Cross, R.F., House, J.A., 1980. Rotavirus-like, calicivirus-like, and 23-nm virus-like particles associated with diarrhea in young pigs. Journal of Clinical Microbiology 12, 105-111.

Saif, L.J., Jiang, B., 1994. Nongroup A rotaviruses of humans and animals. Current Topics in Microbiology & Immunology 185, 339-371.

Scheuer, K.A., Oka, T., Hoet, A.E., Gebreyes, W.A., Molla, B.Z., Saif, L.J., Wang, Q.H., 2013. Prevalence of porcine noroviruses, molecular characterization of emerging porcine sapoviruses from finisher swine in the United States, and unified classification scheme for sapoviruses. Journal of clinical microbiology 51, 2344-2353.

Shan, T.L., Li, L.L., Simmonds, P., Wang, C.L., Moeser, A., Delwart, E., 2011. The fecal virome of pigs on a high-density farm. Journal of virology 85, 11697-11708.

Svensmark, B., 2009. New Neonatal Diarrhoea Syndrome in Denmark. In: Proceedings 1st ESPHM, Faculty of Life Sciences, Copenhagen, Denmark, p. 27.

Vemulapalli, R., Gulani, J., Santrich, C., 2009. A real-time TaqMan RT-PCR assay with an internal amplification control for rapid detection of transmissible gastroenteritis virus in swine fecal samples. Journal of virological methods 162, 231-235.

Verma, H., Mor, S.K., AbdelGlil, M.Y., Goyal, S.M., 2013. Identification and molecular characterization of porcine kobuvirus in U.S. swine. Virus genes 46, 551-553.

Vijgen, L., Moes, E., Keyaerts, E., Li, S., Van Ranst, M., 2008. A pancoronavirus RT-PCR assay for detection of all known coronaviruses. Methods in Molecular Biology 454, 3-12.

Wallgren, P., Mattsson, S., Merza, M., 2012. New neonatal porcine diarrhoea. II. Aspects on etiology. In: Proceedings of the 22nd IPVS Congress. Jeju, Korea, p. 76.

Xiao, C.T., Gimenez-Lirola, L.G., Gerber, P.F., Jiang, Y.H., Halbur, P.G., Opriessnig, T., 2013. Identification and characterization of novel porcine astroviruses (PAstVs) with high prevalence and frequent co-infection of individual pigs with multiple PAstV types. Journal of General Virology 94, 570-582.

Table 1. Samples tested for virus by PCR

Herd	Day	Case animals (n)	Control animals (n)
1	3	7*	6
	5	6	7
2	4	2	2
	5	6	6
	7	3	4
3	4	8	7
	6	6	6
4	3	8	6
	5	0	2
I alt		**46**	**46**

*: One sample was not tested by pan-corona PCR

Table 2. Results of Next Generation Sequencing

Herd	Case animals (n)	Result of test of pools of 5 samples
1	5	Porcine rotavirus (group C) - [1 Contig, 26 reads] Porcine kobuvirus - [6 Contigs, 206 reads]
2	5	Rotavirus (group A) - [1 Contig, 10 reads] Porcine kobuvirus - [1 Contig, 1282 reads]
3	5	Porcine teschovirus - [3 Contigs, 288 reads] Porcine kobuvirus - [16 Contigs, 1479 reads]
4	5	Porcine kobuvirus - [11 Contigs, 262 reads]

Photo: Martin Dam Kristensen

Photo: Martin Dam Kristensen

6 Discussion

The four study herds all presented high standards of housing and hygiene and used proper vaccination protocols against enterotoxigenic *E. coli* and *C. perfringens* type C. Despite this, they all had experienced a high prevalence of neonatal diarrhoea for a long period prior to the investigation. These characteristics, combined with the fact that neither PRRSV nor known diarrhoeal pathogens seemed to cause the problems made them suitable for investigation on the new syndrome NNPDS. Whether or not the diarrhoeal symptoms in the four herds were in fact representative for the new syndrome is difficult to say. Anyway, the remission of symptoms in Herd 4 was inconvenient.

The rationale for choosing a study design without control herds (= herds not clinically affected by neonatal diarrhoea) was for one thing the practical challenge in selecting proper control herds. Since some level of neonatal diarrhoea is a common phenomenon, it would be difficult to categorize a herd as "un-affected." Moreover, since the current studies constituted the starting point in scrutinizing the background of NNPDS it was considered most efficient to start in suspected case herds. We expected that the agent(s) responsible for NNPDS would be more prevalent in diarrhoeic vs. non-diarrhoeic piglets even though some of the non-diarrhoeic piglets were likely to be in the incubation phase of disease.

Antibiotic treatment of piglets and sows may have biased the results of the study. Thus, bacteriological conclusions should take into account that all the older Case piglets (n=20) and none of the Control piglets were medicated prior to euthanasia. Regarding the epidemiological studies, one should keep in mind, that the effect on ADG and mortality by diarrhoea was probably affected by medication of piglets, and that the "true effect" of sow disease may have been underestimated due to medication of sows.

6.1 Gross lesions

Establishing gross pathological hallmarks of NNPDS turned out to be difficult. Intestinal flaccidity, which was the most constant finding in diarrhoeic piglets, is a common finding in cases of neonatal diarrhoea. Grossly, it seems to count for most cases of neonatal diarrhoea that intestines are flaccid with no obvious evidence of inflammation - independent of the underlying aetiology (Brown et al., 2007; Svendsen et al., 1975). Thus, NNPDS seems to fit well into the broad category of so-called "Undifferentiated diarrhoea of neonatal animals" (Brown et al., 2007).

Interestingly, however, all diarrhoeic piglets in the study had milk-filled stomachs at necropsy, indicating that the symptoms were not caused by malabsorption due to insufficient nutrition.

6.2 Histopathology and detection of infectious agents

Villous atrophy with crypt hyperplasia was the predominant histological lesion in diarrhoeic piglets. In suckling piglets villous atrophy is generally associated with viral or parasitic infections (Brown et al., 2007).

However, in this study, the villous atrophy did not appear to be explained by infections with rotavirus A, coronavirus, *Cryptosporidium spp*, *Giardia spp*, *Cystoisospora suis* or *Strongyloides ransomi.*

In order to further investigate the potential role of virus in the syndrome, selected piglets were tested by a range of specific and general tests for the presence of viral agents. Samples from piglets within Herds 1-3 were subjected to PCR- testing on rotavirus A (since we suspected a low sensitivity of the ELISA), rotavirus C, TGEV, PEDV, astrovirus, sapovirus and norovirus. Furthermore, selected case piglets (piglets exhibiting villous atrophy) were tested using pan-viral microarrays, Next generation de novo Sequencing (NGS) and Transmission Electron Microscopy (data presented in Manuscript 4).

The only virus detected by PCR was rotavirus A which was detected in a total of seven piglets – with no obvious association to diarrhoea or villous atrophy. Kobuvirus was detected by NGS in piglets from all herds, whereas rotavirus C and teschovirus was detected in Herd 1 and Herd 3, respectively. A previous study found an association between diarrhoea in suckling piglets and the detection of porcine kobuvirus (Park et al., 2010). In that study, however, co-infections were present in 81% of cases. Interestingly, another study detected a higher rate of kobuvirus in healthy than in diarrhoeic piglets (Shan et al., 2011). Thus, kobuvirus might be a normal part of the microbiota in suckling piglets. In a study by Marthaler et al. (2013) rotavirus C was detected in many cases of neonatal diarrhoea, perhaps indicating a causative association. Since in the current study, rotavirus C was only detected in one of four herds and not by PCR in any piglet, the overall significance of this virus in the syndrome seems limited.

Inflammatory reactions in the intestinal mucosa were associated with diarrhoea in Herd 2, only. The detection of *E. coli* and *Enterococcus spp* in this herd may be causatively linked with the enteritis, although no direct association was detected. Whether *Enterococcus spp* were co-factors, secondary invaders or perhaps primary pathogens in these cases of diarrhoea is an interesting question. A few studies have suggested a primary significance of *Enterococcus spp* in neonatal diarrhoea (Cheon and Chae, 1996; Larsson et al., 2013), but these bacteria are generally considered commensals (Fisher and Phillips, 2009). In the current context it is interesting that a synergetic interaction between *Enterococcus spp* and *E. coli* previously was demonstrated (Lavigne et al., 2008). A similar effect may be a triggering event for enteritis in Herd 2.

Interestingly, villous atrophy was (weakly) associated with the detection of *E. coli* and *Enterococcus spp* within Herd 2 (Jonach et al., 2014). Attaching and effacing *E. coli* have been associated with villous atrophy in neonatal piglets (Helie et al., 1991; Janke et al., 1989), thus, a causative link seems plausible. What seems confusing, though, is the fact that even though *E. coli* bacteria were demonstrated by FISH to adhere to the mucosa, the *E. coli* isolates tested by PCR did not carry any adhesion genes. This discrepancy suggests that other adhesion factors than F4, F5, F6, F18, F41, AIDA-1, intimin and Paa may have been present and responsible for the adherence.

Necrotic lesions were detected in a few piglets from Herd 1 and Herd 4. Apart from *Cp*C, which was detected in both of these herds and which is a well-known cause of necrotic enteritis, necrotic intestinal lesions have been associated with infection with *Cp*A (Nabuurs et al., 1983; Olubunmi and Taylor, 1985) and *Cd* (Keel and Songer, 2006). In this study, neither *Cp*A nor *C. difficile* seemed to be linked with the lesions, but *F. necroforum* was detected by FISH within two of three cases of necrotic lesions. *F. necroforum* was previously associated with necrotic lesions in cases of post-weaning enteritis (Jensen et al., 2008).

The detection of *Cp*C in two of four study herds was unexpected, since *Cp*C is rarely detected in routine laboratory submissions from Danish herds (Svensmark, 2009), and all the sows in the study were vaccinated with commercial vaccines. Also, many international studies do not detect *Cp*C in cases of neonatal diarrhoea (Cruz Jr et al., 2013; Farzan et al., 2013; Gin et al., 2010; Lippke et al., 2011). Overall, necrotic lesions - as well as the detection of *Cp*C - were rare in the study and *Cp*C did not seem to have a primary significance in the outbreaks. However, the unexpected findings underlined the importance of examining animals for more than one day when sampling for diagnostic testing.

In contrast to what was the case for *Cp*C, none of the study herds vaccinated against *Cp*A at the point of investigation. Twice as many Control piglets as Case piglets were *Cp*A positive by culture, thus, a causative significance of this bacterium in NNPDS seems unlikely. The luminal localization of bacteria seemed to support this conclusion, though it is not established whether close proximity to the epithelium is a prerequisite for *Cp*A to cause disease. Some of the bacteriological differences may be explained by the fact that no Control piglets were medicated. However, there was no obvious link between medication and detection of *Cp*A in Case piglets.

The study did not associate the detection of beta2-toxin in small intestinal contents with diarrhoea. This finding is in concordance with a previous study (Farzan et al., 2013). Beta2- toxin being detected in *Cp* culture-negative intestines in the current study is not straightforward to interpret. Since bacteriological culture and toxin detection were not performed on the exact same intestinal segments (culture was performed on a segment of jejunum oral to the segment tested for toxin), the finding could seem to point out local differences in microbial environments. Such differences are not normally taken into account in laboratory examinations, but they may be important when attempting to diagnose *Cp*A-related disease. Potential problems with the performance of tests (lack of sensitivity of the bacteriological culture or lack of specificity of the ELISA) need further consideration in future investigations.

Irrespective of diarrhoeic status of piglets, small amounts of *C. difficile* bacteria were detected within the luminal contents of intestines. Detection of *Cd* toxins A/B, which by some authors is considered the gold standard for diagnosis of *Cd*-related disease (Songer, 2012) was not performed. Toxin detection was not prioritized, since both culture and FISH-methods pointed to a very low prevalence of bacteria. The typical colonic "volcano-lesions" described in *Cd*-related disease (Keel and Songer, 2006) were not detected in any piglet and – apart from one case with colonic intestinal necrosis, the epithelial lesions in colon were mild. Altogether, considering the minimal colonic lesions and the rare detection of *Cd* bacteria in this study, it seems fair to exclude these bacteria as primary agents in the investigated cases of diarrhoea. However, since lesions in cecum have often been associated with *Cd*-related disease (Keel and Songer, 2006; Yaeger et al., 2007) histological examination of this section of intestines should probably have been performed as well.

A general remark regarding herd-associated findings might be in place. Some findings – like small intestinal flaccidity, watery contents in the small intestine and villous atrophy – seemed to be relatively common in non-diarrhoeic piglets within some herds (especially Herd 4 stood out). Regarding infectious agents, clear herd-differences were seen in the detection of *Enterococcus spp*, EAST-1 positive *E. coli* and *Cp*A. In Herd 2, *Enterococcus spp* and *E. coli* may have played a role in disease, whereas in the other herds, they were probably part of the commensal microbiota. The high prevalence of *Cp*A in diarrhoeic piglets from Herd 4, probably reflected that these piglets were in remission of disease, and starting to re-establish a normal

microbiota. Altogether, the important lesson seems to be that herd of origin should always be taken into account when attempting to draw inferences between pathology, microbiology and disease. A recent study by Cruz Jr et al. (2013) underlined this point in relation to mesocolonic edema. In contrast to other authors suggesting this phenomenon to be associated with *Cd*-infection, this study pointed out that the finding was more likely to be a common finding within some herds. Unfortunately, many studies on infectious agents tend to select a few Case pigs in a large amount of herds for diagnostic testing. Such studies are likely to overlook findings of relevance to individual herd-cases or, on the other hand, over-interpret findings that are normal phenomena within some herds.

6.3 Epidemiologic findings

The fact that only four herds were included in the study and that piglets were euthanized during the study-period were obvious limitations in the study design. On the positive side, data obtained in the herds had a great level of detail, thus offered good insights into the development of diarrhoea. Prohibiting cross-fostering offered a unique insight into the epidemiology of diarrhoea, which is impossible to obtain in traditional herd-settings. The relatively low prevalence of diarrhoea in Herd 2 and 3 indicated that some of their previous problems were probably caused by excessive moving of piglets, resulting in deficient colostral immunity and periods of insufficient nutrition.

Litter sizes and the number of stillborn piglets in the herds of the study were comparable to average Danish herds (Vinther, 2013). The birth weights of piglets in the study also fitted well with previous Danish studies in non-problem herds (personal communication, Flemming Thorup, DPRC), and clinical examinations on sows and piglets did not point to general health problems within the herds. Thus, in overall terms the herds of the study did not distinguish themselves from Danish herds in general.

6.4 Risk factors for development of NNPDS

The most important factors associated with the development of NNPDS were herd of origin and sow-parity. Different factors may predispose first parity litters to develop neonatal diarrhoea. In the current context the association might suggest an infectious aetiology of the syndrome, since first parity colostrum contains a lower level of antibodies (Fairbrother and Gyles, 2012). The observed association with herd was mainly explained by a very high prevalence of diarrhoea in Herd 1 and a low prevalence of diarrhoea in Herd 4.

The reason for Herd 1 to stand out from the rest was not explained by the study. Microbiologically, the only finding distinguishing this herd from the others was the detection of rotavirus group C (a pool from five diarrhoeic piglets was positive by NGS, but the virus was not detected in any piglets by PCR). As previously discussed, *Cp*C was also detected in Herd 4, which experienced the mildest outbreak of diarrhoea of the study, thus, this infection did not seem to explain the severity of symptoms in Herd 1. Non-infectious factors like housing or feeding may have contributed to the seriousness of symptoms, though no obvious problems were identified by the local veterinary practitioner or me.

6.5 Effects of NNPDS

In terms of clinical signs of failure to thrive, piglets with NNPDS were debilitated with a high prevalence of hollow flanks, protruding ribs and rough hair coats on the fifth day of life. Comparable clinical registrations in cases of neonatal diarrhoea with other underlying aetiologies are not available. Thus, it is difficult to establish whether NNPDS has a more severe clinical effect than other types of diarrhoea. Generally, herd owners and swine practitioners tend to stress the severity of clinical signs associated with this syndrome. However, the psychological effect of dealing with a problem that does not respond to treatment might affect the way the clinical signs are perceived.

The effects of individual symptoms of NNPDS of 9-14 g ADG reduction were comparable to effects of diarrhoea in a previous Danish study involving the whole suckling period (Johansen et al., 2004). The considerable negative effect of NNPDS was, however, underlined by the fact that a reduction in ADG was also observed in piglets only showing symptoms for a single day. The larger effect seen in piglets belonging to a severely affected litter pointed out that litter-wise diarrhoea was most important in terms of disease but might also be an indication that a diagnosis of diarrhoea is more valid when the whole litter is considered. Compared to a previous Danish study, which detected a reduction in ADG of 14 g per day in diarrhoeic- vs. non-diarrhoeic litters (Svensmark et al., 1989a), the effect of 38 g ADG reduction found in this study was remarkable. Study-designs and statistical methods used in the studies were, however, not directly comparable.

The fact that NNPDS was generally not associated with increased mortality contrasts with previous studies on suckling piglet diarrhoea (Gardner et al., 1989; Lingaas, 1991). However, the finding matches experience from swine practitioners reporting an increase in mortality of 1-5% only, when herds are affected by NNPDS (Kongsted, 2013). Herd 1, experiencing 25% mortality in the diarrhoeic group of piglets seemed to represent a clinical extreme. It is important to stress the disadvantages in study-design with respect to potential effects of NNPDS on mortality. Since piglets suffering from diarrhoea were euthanized during the study-period, the overall mortality in most of the study herds was markedly lower than in normal herd-situations.

Photo: Martin Dam Kristensen

7 Conclusions

Based on fact that well-known agents (ETEC, *Clostridium perfringens* type C, *Clostridium difficile*, rotavirus A, coronavirus (TGEV/ PEDV) and *Cystoisospora suis*) were not associated with piglet diarrhoea in the four selected herds, it was concluded that all herds seemed to represent a so far un-described phenomenon; New Neonatal Porcine Diarrhoea Syndrome (NNPDS).

The syndrome was not associated with starvation. Intestines affected by NNPDS were characterized by flaccidity and no conspicuous signs of inflammation or necrosis. The primary histologic lesion was atrophy of intestinal villi.

The study did not point out any infectious agent causing the syndrome across herds. Within Herds 1, 3 and 4 the prevalence of pathogens was very low, and neither unspecific bacterial FISH probes nor general virus detection methods suggested any obvious explanation on the symptoms. Since kobuvirus was detected in Case piglets from all herds, this virus needs further investigation. Within Herd 2, EAST-1 positive *E. coli* and *Enterococcus spp* were associated with diarrhoea. More Clostridium perfringens type A were detected in non-diarrhoeic than in diarrhoeic piglets.

The most important factors associated with the development of NNPDS were herd of origin and sow-parity. Piglets in Herd 1 had a 12.8 times higher probability of developing NNPDS compared to piglets in Herd 4. Piglets born by first parity sows had a 4.1 higher probability of developing NNPDS than piglets born by mature sows. Birth weight and faecal consistency on the day of birth were minor risk factors.

Depending on the duration of symptoms, NNPDS in individual piglets negatively affected ADG by 9-14 g per day, whereas a diarrhoeic litter-status negatively affected individual piglets by 38 g per day. In piglets only diarrhoeic on the day of birth the ADG was not negatively affected. Within Herd 1, NNPDS seemed to be associated with mortality, whereas in general, the study did not show lethal effects of NNPDS.

It needs to be underlined that the study did not establish if the four herds in fact suffered from the same syndrome. At the current stage, NNPDS is defined by ruling out other causes of neonatal diarrhoea. Though all four herds fulfilled this definition, major differences in the clinical course of disease suggested that Herd 1 was affected by a distinct and more severe condition than the others and the microbiological findings in Herd 2 seemed distinct from findings in the other herds. The lack of control herds in the study complicates the interpretation of findings; however, selecting proper control herds at this stage of investigation was not a straightforward option.

Photo: Martin Dam Kristensen

8 Perspectives

The results in the project offered some practical guidelines for future research on NNPDS. Since liquid faecal consistency day one was common and in many cases was not associated with disease, sampling for laboratory examination should avoid this day. Taking the experience from all four herds together, a reasonable selection protocol in future studies would include 4 to 5 day-old piglets from severely affected litters, having had symptoms for a minimum of two days. The study underlined the importance of selecting a sufficient amount of animals within herds in order to be able to focus on relevant findings within as well as across herds.

Some of the infectious agents detected in the study deserve further attention. In relation to the *E. coli* findings in Herd 2, laser capture micro-dissection and subsequent gene-sequencing seems relevant. Performing infection studies using isolates of *E. coli* and *Enterococcus spp* from Herd 2 may also be rewarding. The potential role of kobuvirus in NNPDS needs further investigation, using PCR on samples from both Case and Control piglets.

Further studies on beta-2 toxin may also be relevant. The surprising findings that beta-2 toxin was detected in piglets that were *Cp* negative by culture – seemed to suggest that diagnostic methods currently used in the research on *Cp*A may be insufficient. Future investigations performing parallel tests on the exact same intestinal segment are needed to get to term with these apparent discrepancies. Immunohistochemical detection of beta-2 toxin in intestinal tissue has previously been performed on horse intestines (Bacciarini et al., 2003), and may also be rewarding in relation to NNPDS. Also, since a previous study using beta-2 ELISAs on intestinal contents did not associate the presence of this toxin with neonatal diarhhoea either (Farzan et al., 2013) other toxins than beta-2 may be relevant to investigate.

Due to the general low prevalence of pathogens in the study, possible non-infectious aetiologies also need to be investigated. The atrophy of villi detected in the study may e.g. be explained by a developmental immaturity rather than a response to infectious agents. As suggested by Sangild et al. (2013), genetic selection pressure for lean meat and large litter sizes could be linked with a decrease in intestinal maturity at birth. Changes in sow-feeding regimens may also have a role to play.

With respect to clinical practice, the study suggested that limiting cross-fostering is probably a good way to start when confronted with a herd case of neonatal diarrhoea. Furthermore, as a starting point, piglets that are diarrhoeic on the day of birth ought not to be medicated, since this is often a normal phenomenon.

Bacteria and viruses did not seem to play an overall role in the investigated outbreaks, thus neither antibiotics nor anti-viral agents seemed indicated. Currently, solutions for oral rehydration might be the best medication in cases of NNPDS. However, despite the fact that laboratory diagnostics do not point to a bacterial aetiology of the syndrome, a beneficial effect of antibiotics cannot be ruled out, since it may prevent secondary invasion by bacteria in the damaged intestinal tissue. Hopefully, future investigations – among these analyses of microbiotas in piglets from the current study (PhD-study by Marie Louise Hermann-Bank,

DTU-Vet) - will point out some useful diagnostic tools in cases of NNPDS and perhaps offer new possibilities for prevention of the syndrome.

9 References

Abid, H.N., Holscher, M.A., Byerly, C.S., 1984. An outbreak of adenovirus enteritis in piglets. Vet Med Sm Anim Clin 79, 105-110.

Alexandersen, S., Knowles, N.J., Dekker, A., Belsham, G.J., Zhang, Z., Koenen, F., 2012. Picornaviruses. In: Zimmerman, J.J., Karriker, L.A., Ramirez, A., Schwartz, K.J., Stevenson, G.W. (Eds.), Diseases of Swine. Wiley-Blackwell, Iowa, USA, pp. 587-620.

Alvarez-Perez, S., Alba, P., Blanco, J.L., Garcia, M.E., 2009a. Detection of toxigenic *Clostridium difficile* in pig feces by PCR. Vet Med-Czech 54, 360-366.

Alvarez-Perez, S., Blanco, J.L., Bouza, E., Alba, P., Gibert, X., Maldonado, J., Garcia, M.E., 2009b. Prevalence of *Clostridium difficile* in diarrhoeic and non-diarrhoeic piglets. Vet Microbiol 137, 302-305.

Amimo, J.O., Vlasova, A.N., Saif, L.J., 2013. Prevalence and genetic heterogeneity of porcine group C rotaviruses in nursing and weaned piglets in Ohio, USA and identification of a potential new VP4 genotype. Veterinary microbiology 164, 27-38.

An, D., Jeoung, H., Jeong, W., Lee, H.S., Park, J., Kim, B., 2011. Porcine kobuvirus from pig stool in Korea. Virus genes 42, 208-211.

Anonymous, 2014. PED Update: Canada Positive. http://www.thepigsite.com/swinenews/35538/ped-update-canada-positive.

Anonymous, 2010. Annual report 2010. http://vsp.lf.dk/~/media/Files/PDF%20-%20Aarsberetning%20VSP%20English/ANNUAL%20REPORT%202010.ashx.

Avbersek, J., Janezic, S., Pate, M., Rupnik, M., Zidaric, V., Logar, K., Vengust, M., Zemljic, M., Pirs, T., Ocepek, M., 2009. Diversity of Clostridium difficile in pigs and other animals in Slovenia. (Special Issue: Clostridium difficile: highlights from Anaerobe 2008.). Anaerobe 15, 252-255.

Bacciarini, L.N., Boerlin, P., Straub, R., Frey, J., Grone, A., 2003. Immunohistochemical localization of Clostridium perfringens beta 2-toxin in the gastrointestinal tract of horses. Vet Pathol 40, 376-381.

Bailey, M., Christoforidou, Z., Grierson, S., Lewis, M., Stokes, C., 2013. Microbiota, metabolism and immunity: the role of early-life events in determining piglet performance. In: Proceedings of the Joint Meeting of the 5th European Symposium of Porcine Health Management and the 50th Anniversary Meeting of the Pig Veterinary Society of Great Britain, Edinburgh, UK.

Bates, D., Maechler, M., Bolker, B., Walker, S., 2013. lme4: Linear mixed-effects models using Eigen and S4. http://CRAN.R-project.org/package=lme4. R package version 1.0-5.

Baums, C.G., Schotte, U., Amtsberg, G., Goethe, R., 2004. Diagnostic multiplex PCR for toxin genotyping of Clostridium perfringens isolates. Vet Microbiol 100:1/2, 11-16.

Brown, C.C., Baker, D.C., Baker, I.K., 2007. Alimentary system. In: Grant Maxie, M. (Ed.), Pathology of Domestic Animals, Vol. 2. Elsevier Saunders, Philadelphia, pp. 1-296.

Bueschel, D.M., Jost, B.H., Billington, S.J., Trinh, H.T., Songer, J.G., 2003. Prevalence of *cpb*2, encoding beta2 toxin, in *Clostridium perfringens* field isolates: correlation of genotype with phenotype. Vet Microbiol 94:2, 121-129.

Byun, J.W., Jung, B.Y., Kim, H.Y., Fairbrother, J.M., Lee, M.H., Lee, W.K., 2013. O-serogroups, virulence genes of pathogenic Escherichia coli and pulsed-field gel electrophoresis (PFGE) patterns of O149 isolates from diarrhoeic piglets in Korea. Vet Med-Czech 58, 468-476.

Cappuccio, J.A., Quiroga, M.A., Moredo, F.A., Canigia, L.F., Machuca, M., Capponi, O., Bianchini, A., Zielinski, G., Sarradell, J., Ibar, M. *and others*, 2009. Neonatal piglets mesocolon edema and colitis due to *Clostridium difficile* infection: prevalence, clinical disease and pathological studies. Braz J Vet Pathol 2, 35-40.

Chan, G., Farzan, A., DeLay, J., McEwen, B., Prescott, J.F., Friendship, R.M., 2013. A retrospective study on the etiological diagnoses of diarrhea in neonatal piglets in Ontario, Canada, between 2001 and 2010. Can J Vet Res 77, 254-260.

Chang, K., Saif, L.J., Kim, Y., 2012. Reoviruses (Rotaviruses and Reoviruses). In: Zimmerman, J.J., Karriker, L.A., Ramirez, A., Schwartz, K.J., Stevenson, G.W. (Eds.), Diseases of Swine. Wiley-Blackwell, Iowa, USA, pp. 621-634.

Chapman, T.A., Wu, X., Barchia, I., Bettelheim, K.A., Driesen, S., Trott, D., Wilson, M., Chin, J.J.C., 2006. Comparison of virulence gene profiles of Escherichia coli strains isolated from healthy and diarrheic swine. Appl Environ Microbiol 72:7, 4782-4795.

Cheetham, S., Souza, M., Meulia, T., Grimes, S., Han, M.G., Saif, L.J., 2006. Pathogenesis of a genogroup II human norovirus in gnotobiotic pigs. J Virol 80, 10372-10381.

Cheon, D., Chae, C., 1996. Outbreak of diarrhea associated with Enterococcus durans in piglets. J Vet Diagn Invest 8, 123-124.

Choi, C., Cho, W., Chung, H., Jung, T., Kim, J., Chae, C., 2001. Prevalence of the enteroaggregative Escherichia coli heat-stable enterotoxin 1 (EAST1) gene in isolates in weaned pigs with diarrhea and/or edema disease. Vet microbiol 81, 65-71.

Collins, J.E., Bergeland, M.E., Bouley, D., Ducommun, A.L., Francis, D.H., Yeske, P., 1989. Diarrhea associated with *Clostridium perfringens* type A enterotoxin in neonatal pigs. J Vet Diagn Invest 1:4, 351-353.

CoreTeam, R., 2013. R: A language and environment for statistical computing. R foundation for statistical computing, Vienna, Austria. URL http://WWW.R-project.org/.

Costinar, L., Pascu, C., Herman, V., Panici, G., Balean, F., Spiru, D., Marecec, C., Pava, S., 2011. Investigation of digestive disorder's etiology in piglets from farms in west of Romania. Lucrari Stiintifice - Universitatea de Stiinte Agricole a Banatului Timisoara, Medicina Veterinara 44, 167-172.

Cruz Jr, E.C., Salvarani, F.M., Silva, R.O.S., Silva, M.X., Lobato, F.C.F., Guedes, R.M.C., 2013. A surveillance of enteropathogens in piglets from birth to seven days of age in Brazil. Pesquisa Vet Brasil 33, 963-969.

Damme-Jongsten, M., Haagsma, J., Notermans, S., 1990. Testing strains of Clostridium perfringens type A isolated from diarrhoeic piglets for the presence of the enterotoxin gene. Vet Rec 126, 191-192.

de la Fé Rodríguez, P.Y., Maroto Martin, L.O., Cruz Munoz, E., Imberechts, H., Butaye, P., Goddeeris, B.M., Cox, E., 2013. Several enteropathogens are circulating in suckling and newly weaned piglets suffering from diarrhea in the province of Villa clara, Cuba. Trop Anim Health Pro 45:2, 435-440.

Didier, E.S., Didier, P.J., Snowden, K.F., Shadduck, J.A., 2000. Microsporidiosis in mammals. Microbes Infect 2, 709-720.

Ducatelle, R., Coussement, W., Hoorens, J., 1982. Sequential pathological study of experimental porcine adenovirus enteritis. Vet pathol 19, 179-189.

Enemark, H.L., Ahrens, P., Bille-Hansen, V., Heegaard, P.M.H., Vigre, H., Thamsborg, S.M., Lind, P., 2003. Cryptosporidium parvum: infectivity and pathogenicity of the 'porcine' genotype. Parasitology 126:5, 407-416.

Estrada Correa, A.E., Taylor, D.J., 1989. Porcine Clostridium perfringens type A spores, enterotoxin and antibody to enterotoxin. Vet Rec 124, 606-610.

Eysker, M., Boerdam, G.A., Hollanders, W., Verheijden, J.H.M., 1994. The prevalence of Isospora suis and Strongyloides ransomi in suckling piglets in the Netherlands. Veterinary Quarterly 16, 203-205. 13 ref.

Fairbrother, J.M., Gyles, C.L., 2012. Colibacillosis. In: Zimmerman, J.J., Karriker, L.A., Ramirez, A., Schwartz, K.J., Stevenson, G.W. (Eds.), Diseases of Swine. Wiley-Blackwell, Iowa, USA, pp. 723-749.

Farzan, A., Kircanski, J., DeLay, J., Soltes, G., Songer, J.G., Friendship, R., Prescott, J.F., 2013. An investigation into the association between cpb2-encoding Clostridium perfringens type A and diarrhea in neonatal piglets. Can J Vet Res 77:1, 45-53.

Feasey, N.A., Healey, P., Gordon, M.A., 2011. The aetiology, investigation and management of diarrhoea in the HIV-positive patient. Aliment Pharm Ther 34, 587-603.

Fisher, K., Phillips, C., 2009. The ecology, epidemiology and virulence of Enterococcus. Microbiology 155, 1749-1757.

Flynn, W.T., Saif, L.J., Moorhead, P.D., 1988. Pathogenesis of porcine enteric calicivirus-like virus in four-day-old gnotobiotic pigs. Am J Vet Res 49, 819-825.

Frydendahl, K., 2002. Prevalence of serogroups and virulence genes in *Escherichia coli* associated with postweaning diarrhoea and oedema disease in pigs and a comparison of diagnostic approaches. Vet Microbiol 85:2, 169-182.

Gardner, I.A., Hird, D.W., Franti, C.E., 1989. Neonatal survival in swine: effects of low birth weight and clinical disease. American Journal of Veterinary Research; 1989.50: 5, 792-797.24 ref .

Gelberg, H.B., Woode, G.N., Kniffen, T.S., Hardy, M., Hall, W.F., 1991. The shedding of group A rotavirus antigen in a newly established closed specific pathogen-free swine herd. Vet Microbiol 28, 213-229.

Geurden, T., Vercruysse, J., Claerebout, E., 2010. Is Giardia a significant pathogen in production animals? (Special Issue: Cryptosporidium and other waterborne protozoa.). Exp Parasitol 124, 98-106.

Gibert, M., Jolivet-Renaud, C., Popoff, M.R., 1997. Beta2 toxin, a novel toxin produced by Clostridium perfringens. Gene 203, 65-73.

Gin, T., Guennec, J.L., Morvan, H., Martineau, G.P., 2010.
Clinical and laboratory investigations in 10 French pig herds dealing with enzootic neonatal diarrhea. In: Proceedings of the 21st IPVS Congres, Vancouver, Canada, pp. 758.

Greve, J.H., 2012. Internal parasites: Helminths. In: Zimmerman, J.J., Karriker, L.A., Ramirez, A., Schwartz, K.J., Stevenson, G.W. (Eds.), Diseases of Swine, pp. 908-909-920.

Guo, M., Hayes, J., Cho, K.O., Parwani, A.V., Lucas, L.M., Saif, L.J., 2001. Comparative pathogenesis of tissue culture-adapted and wild-type Cowden porcine enteric calicivirus (PEC) in gnotobiotic pigs and induction of diarrhea by intravenous inoculation of wild-type PEC. J Virol 75, 9239-9251.

Gyles, G.L., Fairbrother, J.M., 2004. Escherichia coli. In: Gyles, C.L., Prescott, J.F., Songer, J.G., Thoen, C.O. (Eds.), Pathogenesis of Bacterial Infections in Animals, pp. 193-223.

Ha, S.K., Choi, C., Jung, K., Kim, J., Han, D.U., Ha, Y., Lee, S.D., Kim, S.H., Chae, C., 2004. Genotypic prevalence of the adhesin involved in diffuse adherence in Escherichia coli isolates in pre-weaned pigs with diarrhoea in Korea. J Vet Med B 51, 166-168.

Hamnes, I.S., Gjerde, B.K., Forberg, T., Robertson, L.J., 2007. Occurrence of *Cryptosporidium* and *Giardia* in suckling piglets in Norway. Vet Parasitol 144, 222-233.

Haugegaard, J., 2010. Prevalence of nematodes in Danish industrialized sow farms with loose housed sows in dynamic groups. Vet Parasitol 168, 156-159.

Helie, P., Morin, M., Jacques, M., Fairbrother, J.M., 1991. Experimental infection of newborn pigs with an attaching and effacing Escherichia coli O45:K"E65" strain. Infect Immun 59:3, 814-821.

Hindsbo, O., Nielsen, C.V., Andreassen, J., Willingham, A.L., Bendixen, M., Nielsen, M.A., Nielsen, N.O., 2000. Age-dependent occurrence of the intestinal ciliate Balantidium coli in pigs at a Danish research farm. Acta Vet Scand 41, 79-83.

Jacobi, S.K., Odle, J., 2012. Nutritional factors influencing intestinal health of the neonate. Adv Nutr 3, 687-696.

Janke, B.H., Francis, D.H., Collins, J.E., Libal, M.C., Zeman, D.H., Johnson, D.D., 1989. Attaching and effacing Escherichia coli infections in calves, pigs, lambs, and dogs. J Vet Diagn Invest 1:1, 6-11.

Janke, B.H., Morehouse, L.G., Solorzano, R.F., 1988. Single and mixed infections of neonatal pigs with rotaviruses and enteroviruses: clinical signs and microscopic lesions. Can J Vet Res 52, 364-369.

Janke, B.H., Nelson, J.K., Benfield, D.A., Nelson, E.A., 1990. Relative prevalence of typical and atypical strains among rotaviruses from diarrheic pigs in conventional swine herds. J Vet Diagn Invest 2:4, 308-311.

Jenikova, M., Nemejc, K., Sak, B., Kvetonova, D., Kvac, M., 2011. New view on the age-specificity of pig Cryptosporidium by species-specific primers for distinguishing Cryptosporidium suis and Cryptosporidium pig genotype II. Vet Parasitol 176, 120-125.

Jensen, T.K., Haugegaard, S., Boye, M., 2008. Demonstration of *Fusobacterium necroforum*-associated necrotizing enterocolitis by flourescent In sity Hybridization in weaned pigs. In: Proceedings of the Concurrent Meetings of the ACVP and ASVCP, San Antonio, Texas, pp. 772.

Jeong, D., Won, G.P., B-K., Hur, J., You, J., Kang S-J., Oh I-G., Lee, Y., Stein, B.D., Lee, J.H., 2007. Occurrence and genotypic characteristics of Enterocytozoon bieneusi in pigs with diarrhea. Parasitol Res 102, 123-128.

Johannsen, U., Arnold, P., Kohler, B., Slebitz, H.J., 1993a. Experimental Clostridium perfringens type A enterotoxaemia in unweaned piglets. Monatsh Veterinarmed 48:3, 129-136.

Johannsen, U., Menger, S., Arnold, P., Kähler, B., Selbitz, H.J., 1993b. Experimental Clostridium perfringens type A enterotoxaemia in unweaned piglets. II. Light- and electron- microscopic investigations on the pathology and pathogenesis of experimental C. perfringens type A infection. Monatsh Veterinarmed 48:6, 299-306.

Johansen, M., Alban, L., Kjaersgard, H.D., Baekbo, P., 2004. Factors associated with suckling piglet average daily gain. Preventive veterinary medicine 63, 91-102.

Jonach, B., Boye, M., Stockmarr, A., Jensen, T., 2014. Fluorescence in situ hybridization investigation of potentially pathogenic bacteria involved in neonatal porcine diarrhea. BMC Vet Res 10, 68.

Katsuda, K., Kohmoto, M., Kawashima, K., Tsunemitsu, H., 2006. Frequency of enteropathogen detection in suckling and weaned pigs with diarrhea in Japan. J Vet Diagn Invest 18, 350-354.

Keel, M.K., Songer, J.G., 2006. The Comparative Pathology of Clostridium difficile-associated Disease. Vet Pathol 43, 225-240.

Khamrin, P., Maneekarn, N., Hidaka, S., Kishikawa, S., Ushijima, K., Okitsu, S., Ushijima, H., 2010. Molecular detection of kobuvirus sequences in stool samples collected from healthy pigs in Japan. Infect Genet Evol 10, 950-954.

Khamrin, P., Maneekarn, N., Kongkaew, A., Kongkaew, S., Okitsu, S., Ushijima, H., 2009. Porcine kobuvirus in piglets, Thailand. Emerg Infect Dis 15, 2075-2076.

Klaasen, H., Molkenboer, M., Bakker, J., Miserez, R., Hani, H., Frey, J., Popoff, M.R., Bosch, J.F., 1999. Detection of the beta 2 toxin gene of *Clostridium perfringens* in diarrhoeic piglets in the Netherlands and Switzerland. FEMS immunol Med Mic 24, 325-332.

Kondova, I., Mansfield, K., Buckholt, M.A., Stein, B., Widmer, G., Carville, A., Lackner, A., Tzipori, S., 1998. Transmission and serial propagation of Enterocytozoon bieneusi from humans and rhesus macaques in gnotobiotic piglets. Infect Immun 66, 5515-5519.

Kongsted, H., 2013. Spædgrisediarré i Danmark annno 2013. http://vsp.lf.dk/~/media/Files/PDF%20-%20Publikationer/Erfaringer%202013/Erfaring_1320_Sp%C3%A6dgrisediarre_i_Danmark_anno_2013.ashx.

Kvac, M., Kestranova, M., Pinkova, M., Kvetonova, D., Kalinova, J., Wagnerova, P., Kotkova, M., Vitovec, J., Ditrich, O., McEvoy, J. *and others*, 2013. Cryptosporidium scrofarum n. sp. (Apicomplexa: Cryptosporidiidae) in domestic pigs (Sus scrofa). Vet Parasitol 191, 218-227.

Kvac, M., Hanzlikova, D., Sak, B., Kvetonova, D., 2009. Prevalence and age-related infection of Cryptosporidium suis, C. muris and Cryptosporidium pig genotype II in pigs on a farm complex in the Czech Republic. Vet Parasitol 160, 319-322.

Langkjaer, R.B., Vigre, H., Enemark, H.L., MaddoxHyttel, C., 2007. Molecular and phylogenetic characterization of Cryptosporidium and Giardia from pigs and cattle in Denmark. Parasitology 134, 339-350.

Larsson, J., Grandon, R., Lindberg, R., Aspan, A., Jacobson, M., 2013. Neonatal porcine diarrhoea associated with small intestinal colonisation of Enterococcus spp. In: Proceedings of the Joint Meeting of the 5th European Symposi-

um of Porcine Health Management and the 50th Anniversary Meeting of the Pig Veterinary Society of Great Britain, Edinburgh, UK.

Lavigne, J.P., NicolasChanoine, M.H., Bourg, G., Moreau, J., Sotto, A., 2008. Virulent synergistic effect between Enterococcus faecalis and Escherichia coli assayed by using the Caenorhabditis elegans model. PLos One 3:10, e3370.

Lewis, M., Inman, C.F., Bailey, M., 2010. Review: postnatal development of the mucosal immune system and consequences on health in adulthood. Can J Anim Sci 90, 129-136.

Lindsay, D.S., Dubey, J.P., Santín-Durán, M., Fayer, R., 2012. Coccidia and other protozoa. In: Zimmerman, J.J., Karriker, L.A., Ramirez, A., Schwartz, K.J., Stevenson, G.W. (Eds.), Diseases of Swine. Wiley-Blackwell, Iowa, USA, pp. 895-907.

Lingaas, F., 1991. Epidemiological and genetical studies in Norwegian pig herds. IV. Breed effects, recurrence of disease, and relationship between disease and some performance traits. Acta Veterinaria Scandinavica 32, 107-114.

Lippke, R.T., Borowski, S.M., Marques, S.M.T., Paesi, S.O., Almeida, L.L., Moreno, A.M., Corbellini, L.G., Barcellos, D.E.S.N., 2011. Matched case-control study evaluating the frequency of the main agents associated with neonatal diarrhea in piglets. Pesquisa Vet Brasil 31, 505-510.

Liu, Z., Li, J., Pan, H., 2014. Seroprevalence and molecular detection of porcine sapovirus in symptomatic suckling piglets in Guangdong Province, China. Trop Anim Health Prod 46, 583-587. 28 ref.

Lvarado G, J., Gonzalez Z, A., Gillman, R., Lopez U., T, 2009. Experimental infection of one-month-old piglets with spores of Enterozytozoon bieneusi. Revista de Investigaciones Veterinarias del Peru (RIVEP) 20, 291-296. 17 ref.

MacLachlan, N.J., Duboi, E.J. (Eds.), 2011. Fenner's Veterinary Virology.

MaddoxHyttel, C., Langkjaer, R.B., Enemark, H.L., Vigre, H., 2006. *Cryptosporidium* and *Giardia* in different age groups of Danish cattle and pigs - occurrence and management associated risk factors. Vet Parasitol 141, 48-59.

Manners, M.J., 1976. The development of digestive function in the pig. Proc Nutr Soc 35, 49-55.

Martelli, P., Lavazza, A., Nigrelli, A.D., Merialdi, G., Alborali, L.G., Pensaert, M.B., 2008. Epidemic of diarrhoea caused by porcine epidemic diarrhoea virus in Italy. Vet Rec 162, 307-310.

Marthaler, D., Rossow, K., Culhane, M., Collins, J., Goyal, S., Ciarlet, M., Matthijnssens, J., 2013. Identification, phylogenetic analysis and classification of porcine group C rotavirus VP7 sequences from the United States and Canada. Virology 446, 189-198.

Marthaler, D., Rossow, K., Gramer, M., Collins, J., Goyal, S., Tsunemitsu, H., Kuga, K., Suzuki, T., Ciarlet, M., Matthijnssens, J., 2012. Detection of substantial porcine group B rotavirus genetic diversity in the United States, resulting in a modified classification proposal for G genotypes. Virology 433, 85-96.

Martins, M., Martinez-Rossi, N., Ferreira, A., Brocchi, M., Yano, T., Castro, A.F.P., Silveira, W.D., 2000. Pathogenic characteristics of Escherichia coli strains isolated from newborn piglets with diarrhea in Brazil. Vet Microbiol 76, 51-59.

Medici, K.C., Barry, A.F., Alfieri, A.F., Alfieri, A.A., 2011. Porcine rotavirus groups A, B, and C identified by polymerase chain reaction in a fecal sample collection with inconclusive results by polyacrylamide gel electrophoresis. J Swine Health Prod 19, 146-150.

Melin, L., Wallgren, P., Mattsson, S., Stampe, M., Lofstedt, M., 2010. Neonatal diarrhoea in piglets from E.coli vaccinated sows in Sweden. In: Proceedings of the 21st IPVS Congress, Vancouver, Canada, p. 290.

Miyazaki, A., Fukuda, M., Kuga, K., Takagi, M., Tsunemitsu, H., 2010. Prevalence of antibodies against transmissible gastroenteritis virus and porcine respiratory coronavirus among pigs in six regions in Japan. J Vet Med Sci 72, 943-946.

Mor, S.K., Chander, Y., Marthaler, D., Patnayak, D.P., Goyal, S.M., 2012. Detection and molecular characterization of Porcine astrovirus strains associated with swine diarrhea. J Vet Diagn Invest 24, 1064-1067.

Morin, M., Magar, R., Robinson, Y., 1990. Porcine group C rotavirus as a cause of neonatal diarrhea in a Quebec swine herd. Can J Vet Res 54, 385-389.

Morin, M., Turgeon, D., Jolette, J., Robinson, Y., Phaneuf, J.B., Sauvageau, R., Beauregard, M., Teuscher, E., Higgins, R., Lariviere, S., 1983. Neonatal diarrhea of pigs in Quebec: infectious causes of significant outbreaks. Canadian Journal of Comparative Medicine 47, 11-17. 38 ref.

Nabuurs, M.J.A., Haagsma, J., Molen, E. J. van der, Heijden, P.J., 1983. Diarrhea in one to three week-old piglets associated with Clostridium perfringens type A. Ann Rech Vet 14:4, 408-411.

Nagy, B., Fekete, P.Z., 1999. Enterotoxigenic Escherichia coli (ETEC) in farm animals. (Special issue. Pathogenic Escherichia coli in animals). Vet Res 30:2/3, 259-284.

Ngeleka, M., Pritchard, J., Appleyard, G., Middleton, D.M., Fairbrother, J.M., 2003. Isolation and association of *Escherichia coli* AIDA-I/STb, rather than EAST1 pathotype, with diarrhea in piglets and antibiotic sensitivity of isolates. J Vet Diagn Invest 15, 242-252.

Ojeniyi, B., Ahrens, P., Meyling, A., 1994. Detection of fimbrial and toxin genes in Escherichia coli and their prevalence in piglets with diarrhoea. The application of colony hybridization assay, polymerase chain reaction and phenotypic assays. J Vet Med B 41, 49-59.

Olubunmi, P.A., Taylor, D.J., 1985. Clostridium perfringens type A in enteric diseases of pig. Trop Veterinarian 3:1/4, 28-33.

Park, S., Kim, H., Moon, H., Song, D., Rho, S., Han, J., Nguyen, V., Park, B., 2010. Molecular detection of porcine kobuviruses in pigs in Korea and their association with diarrhea. Arch Virol 155, 1803-1811.

Pearson, G.R., McNulty, M.S., 1977. Pathological changes in the small intestine of neonatal pigs infected with a pig reovirus-like agent (rotavirus). J Comp Pathol 87, 363-375.

Popoff, M.R., Jestin, A., 1985. Enteropathogenicity of purified Clostridium perfringens enterotoxin in the pig. Am J Vet Res 46:10, 2147-2148.

Reuter, G., Boldizsar, A., Pankovics, P., 2009. Complete nucleotide and amino acid sequences and genetic organization of porcine kobuvirus, a member of a new species in the genus Kobuvirus, family Picornaviridae. Arch Virol 154, 101-108.

Reuter, G., Boros, A., Pankovics, P., 2011. Kobuviruses - a comprehensive review. Rev Medical Virol 21, 32-41.

Reuter, G., Kecskemeti, S., Pankovics, P., 2010a. Evolution of porcine kobuvirus infection, Hungary. Emerg Infect Dis 16, 696-698.

Reuter, G., ZimsekMijovski, J., PoljsakPrijatelj, M., Bartolo, I.d., Ruggeri, F.M., Kantala, T., Maunula, L., Kiss, I., Kecskemeti, S., Halaihel, N. *and others*, 2010b. Incidence, diversity, and molecular epidemiology of sapoviruses in swine across Europe. J Clin Microbiol 48, 363-368.

Roepstorff, A., Jorsal, S.E., 1989. Prevalence of helminth infections in swine in Denmark. Vet Parasitol 33, 231-239.

Roepstorff, A., Nilsson, O., Oksanen, A., Gjerde, B., Richter, S.H., Ortenberg, E., Christensson, D., Martinsson, K.B., Bartlett, P.C., Nansen, P. *and others*, 1998. Intestinal parasites in swine in the Nordic countries: prevalence and geographical distribution. Vet Parasitol 76, 305-319.

Roepstorff, A., 1991. Transmission of intestinal helminths in Danish sow herds. Vet Parasitol 39, 149-160.

Saif, L.J., Bohl, E.H., Theil, K.W., Cross, R.F., House, J.A., 1980. Rotavirus-like, calicivirus-like, and 23-nm virus-like particles associated with diarrhea in young pigs. J Clin Microbiol 12, 105-111.

Saif, L.J., Pensaert, M.B., Sestak, K., Yeo, S., Jung, K., 2012. Coronaviruses. In: Zimmerman, J.J., Karriker, L.A., Ramirez, A., Schwartz, K.J., Stevenson, G.W. (Eds.), Diseases of Swine. Wiley-Blackwell, Iowa, USA, pp. 501-524.

Sanford, S.E., Hoover, D.M., 1983. Enteric adenovirus infection in pigs. Can J Comparat Med 47, 396-400.

Sangild, P.T., Thymann, T., Schmidt, M., Stoll, B., Burrin, D.G., Buddington, R.K., 2013. The preterm pig as a model in pediatric gastroenterology. Journal of animal science 91, 4713-4729.

Sangild, P.T., 2001. Transitions in the life of the gut at birth. In: Digestive Physiology in Pigs. Proceedings of the 8th Symposium, Swedish University of Agricultural Sciences, Uppsala, Sweden, pp. 3-17.

Santin, M., 2013. Clinical and subclinical infections with Cryptosporidium in animals. New Zealand veterinary journal 61, 1-10. many ref.

Savarino, S.J., Fasano, A., Robertson, D.C., Levine, M.M., 1991. Enteroaggregative Escherichia coli elaborate a heat-stable enterotoxin demonstrable in an in vitro rabbit intestinal model. J Clin Invest 87, 1450-1455.

Sayeed, S., Uzal, F.A., Fisher, D.J., Saputo, J., Vidal, J.E., Chen, Y., Gupta, P., Rood, J.I., McClane, B.A., 2008. Beta toxin is essential for the intestinal virulence of Clostridium perfringens type C disease isolate CN3685 in a rabbit ileal loop model. Mol Microbiol 67, 15-30.

Schuster, F.L., RamirezAvila, L., 2008. Current world status of Balantidium coli. Clinical microbiology reviews 21, 626-638.

Shan, T.L., Li, L.L., Simmonds, P., Wang, C.L., Moeser, A., Delwart, E., 2011. The fecal virome of pigs on a high-density farm. Journal of virology 85, 11697-11708.

Shimizu, M., Shirai, J., Narita, M., Yamane, T., 1990. Cytopathic astrovirus isolated from porcine acute gastroenteritis in an established cell line derived from porcine embryonic kidney. J Clin Mcrobiol 28, 201-206.

Silva, R.O.S., Lobato, F.C.F., Pires, P.S., Gabardo, M.P., Paladino, E.S., Guedes, R.M.C., 2013. Neonatal diarrhea in piglets associated with cpb-2 positive Clostridium perfringens. Braz J Vet Pathol 6:1, 11-14.

Silva, R.O.S., Salvarani, F.M., Cruz Junior, E. C. da C., Pires, P.S., Santos, R.L.R., Assis, R.A.d., Guedes, R. M. de C., Lobato, F.C.F., 2011. Detection of enterotoxin A and cytotoxin B, and isolation of *Clostridium difficile* in piglets in Minas Gerais, Brazil. Cienc Rural 41, 1430-1435.

Snowden, K.F., Didier, E.S., Orenstein, J.M., Shadduck, J.A., 1998. Animal models of human microsporidial infections. Lab Anim Sci 48, 589-592.

Songer, J.G., Post, K.W., Larson, D.J., Jost, B.H., Glock, R.D., 2000. Infection of neonatal swine with *Clostridium difficile*. Swine Health Prod 8, 185-189.

Songer, J.G., Uzal, F.A., 2005. Clostridial enteric infections in pigs. J Vet Diagn Invest 17, 528-536.

Songer, J.G., 2012. Clostridiosis. In: Zimmerman, J.J., Karriker, L.A., Ramirez, A., Schwartz, K.J., Stevenson, G.W. (Eds.), Diseases of Swine. Wiley-Blackwell, Iowa, USA, pp. 709-722.

Steele, J., Feng, H., Parry, N., Tzipori, S., 2010. Piglet models of acute or chronic Clostridium difficile illness. J Infect Dis 201, 428-434.

Steele, J., Mukherjee, J., Parry, N., Tzipori, S., 2013. Antibody against TcdB, but not TcdA, prevents development of gastrointestinal and systemic Clostridium difficile disease. J Infect Dis 207, 323-330.

Stevenson, G.W., Hoang, H., Schwartz, K.J., Burrough, E.R., Sun, D., Madson, D., Cooper, V.L., Pillatzki, A., Gauger, P., Schmitt, B.J. *and others*, 2013. Emergence of Porcine epidemic diarrhea virus in the United States: clinical signs, lesions, and viral genomic sequences. Journal of Veterinary Diagnostic Investigation 25, 649-654.

Stewart, T.B., Stone, W.M., Marti, O.G., 1976. Strongyloides ransomi: Prenatal and transmammary infection of pigs of sequential litters from dams experimentally exposed as weanlings. Am J Vet Res 37, 541-544.

Stuart, B.P., Gosser, H.S., Allen, C.B., Bedell, D.M., 1982. Coccidiosis in swine: dose and age response to Isospora suis. Can J Comparat Med 46, 317-320.

Svendsen, J., Bille, N., Nielsen, N.C., Larsen, J.L., Riising, H.J., 1975. Preweaning mortality in pigs. 4. Diseases of the gastrointestinal tract in pigs. Nord Vet Med 27, 85-101.

Svensmark, B., 2009. New Neonatal Diarrhoea Syndrome in Denmark. In: Proceedings 1st ESPHM, Faculty of Life Sciences, Copenhagen, Denmark, pp. 27.

Svensmark, B., Jorsal, S.E., Nielsen, K., Willeberg, P., 1989a. Epidemiological studies of piglet diarrhoea in intensively managed Danish sow herds. I. Pre-weaning diarrhoea. Acta Veterinaria Scandinavica 30, 43-53.

Svensmark, B., Nielsen, K., Dalsgaard, K., Willeberg, P., 1989b. Epidemiological studies of piglet diarrhoea in intensively managed Danish sow herds. III. Rotavirus infection. Acta Vet Scand 30, 63-70.

Theil, K.W., Saif, L.J., Moorhead, P.D., Whitmoyer, R.E., 1985. Porcine rotavirus-like virus (group B rotavirus): characterization and pathogenicity for gnotobiotic pigs. J Clin Microbiol 21:3, 340-345.

Thomson, J.R., Friendship, R.M., 2012. Digestive system. In: Zimmerman, J.J., Karriker, L.A., Ramirez, A., Schwartz, K.J., Stevenson, G.W. (Eds.), Diseases of Swine. Wiley-Blackwell, Iowa, USA, pp. 199-226.

Uzal, F.A., Vidal, J.E., McClane, B.A., Gurjar, A.A., 2010. Clostridium perfringens toxins involved in mammalian veterinay diseases. Open Toxinol J 3, 24-42.

Valencakova, A., Balent, P., Huska, M., Novotny, F., Luptakova, L., 2006. First report on Encephalitozoon intestinalis infection of pigs in Europe. Acta Vet Hung 54, 407-411.

van der Poel, W., van der Vinje, J., van der Heide, R., Herrera, M.I., Vivo, A., Koopmans, M.P.G., 2000. Norwalk-like calicivirus genes in farm animals. Emerg Infect Dis 6, 36-41.

Vancanneyt, M., Snauwaert, C., Cleenwerck, I., Baele, M., Descheemaeker, P., Goossens, H., Pot, B., Vandamme, P., Swings, J., Haesebrouck, F. *and others*, 2001. Enterococcus villorum sp. nov., an enteroadherent bacterium associated with diarrhoea in piglets. Int J Syst Evol Microbiol 51, 393-400.

Verma, H., Mor, S.K., AbdelGlil, M.Y., Goyal, S.M., 2013. Identification and molecular characterization of porcine kobuvirus in U.S. swine. Virus genes 46, 551-553.

Vijgen, L., Moes, E., Keyaerts, E., Li, S., Van Ranst, M., 2008. A pancoronavirus RT-PCR assay for detection of all known coronaviruses. Methods in Molecular Biology 454, 3-12.

Vinther, J., 2013. Landsgennemsnit for produktivitet i svineproduktionen 2012. http://vsp.lf.dk/~/media/Files/PDF%20-%20Publikationer/Notater%202013/Notat_1314 Landsgennemsnit %20for%20produktivitet%20i%20svineproduktionen%202012.ashx.

Vitovec, J., Hamadejova, K., Landova, L., Kvac, M., Kvetonova, D., Sak, B., 2006. Prevalence and pathogenicity of *Cryptosporidium suis* in pre- and post-weaned pigs. J Vet Med B 53, 239-243.

Vu-Khac, H., Holoda, E., Pilipcinec, E., Blanco, M., Blanco, J.E., Dahbi, G., Mora, A., Lopez, C., Gonzalez, E.A., Blanco, J., 2007. Serotypes, virulence genes, intimin types and PFGE profiles of Escherichia coli isolated from piglets with diarrhoea in Slovakia. Vet J 174:1, 176-187.

Wang, Q.H., Han, M.G., Cheetham, S., Souza, M., Funk, J.A., Saif, L.J., 2005. Porcine noroviruses related to human noroviruses. (Special issue: Zoonotic diseases.). Emerg Infect Dis 11, 1874-1881.

Wang, C., Lan, D., Hua, X., 2011. Porcine kobuvirus from pig stool specimens in Shanghai, China. Virus genes 43, 350-352.

Wieler, L.H., Ilieff, A., Herbst, W., Bauer, C., Vieler, E., Bauerfeind, R., Failing, K., Klos, H., Wengert, D., Baljer, G. *and others*, 2001. Prevalence of enteropathogens in suckling and weaned piglets with diarrhoea in southern Germany. J Vet Med B 48, 151-159.

Yaeger, M., Funk, N., Hoffman, L., 2002. A survey of agents associated with neonatal diarrhea in Iowa swine including Clostridium difficile and porcine reproductive and respiratory syndrome virus. J Vet Diagn Invest 14, 281-287.

Yaeger, M.J., Kinyon, J.M., Glenn Songer, J., 2007. A prospective, case control study evaluating the association between Clostridium difficile toxins in the colon of neonatal swine and gross and microscopic lesions. J Vet Diagn Invest 19, 52-59.

Yang, Y., Zeng, L., Li, M., Zhou, J., 1995. Diarrhoea in piglets and monkeys experimentally infected with Balantidium coli isolated from human faeces. J Trop Med Hyg 98, 69-72.

Yu, J., Jin, M., Qing, Z., Li, H., Li, D., Xu, Z., Li, J., Cui, S., Yang, S., Liu, N. *and others*, 2009. Candidate porcine Kobuvirus, China. Emerg Infect Dis 15, 823-825.

Zajacova, Z.S., Konstantinova, L., Alexa, P., 2012. Detection of virulence factors of Escherichia coli focused on prevalence of EAST1 toxin in stool of diarrheic and non-diarrheic piglets and presence of adhesion involving virulence factors in astA positive strains. Vet Microbiol 154:3/4, 369-375.

Zhang, W.P., Zhao, M.J., Ruesch, L., Omot, A., Francis, D., 2007. Prevalence of virulence genes in Escherichia coli strains recently isolated from young pigs with diarrhea in the US. Vet Microbiol 123:1/3, 145-152.

Photo: Martin Dam Kristensen

10 Appendix 1

Dear practitioner,

Herds with neonatal diarrhoea are needed!

The Danish Pig Research Centre and The National Veterinary Institute have started up a research project on "New Neonatal Diarrhoea" – which so far means diarrhea in neonatal pigs not caused by Clostridium perfringens tye C, known E. coli types or rotavirus. The aim of the project is to investigate if there is a common (so far unknown) disease-agent in these herds and to clarify the epidemiological development of disease.

Therefore, I am looking for herds which for a longer period have had problems on neonatal diarrhoea without known cause and which have an interest in participating in a study on the problem.

The following criteria should be met:

- Production herd with a maximum of 30% gilts
- Well-managed herd with stabile and well-trained personnel
- High-prevalent and continuous problems with diarrhea in piglets of 1-3 days of age
- Low effect of antibiotics
- Sow-feed has been evaluated with respect to hygiene and composition
- Mistakes in relation to the piglets' climatic conditions have been taken care of
- No other disease problems in the farrowing unit

Herds in close proximity to Kjellerup will be preferred, since laboratory examinations will be carried out there. However, do not hesitate to contact me if you know a suitable herd not close to Kjellerup.

Hanne Kongsted, Danish Pig Research Centre

Photo: Martin Dam Kristensen

DEPARTMENT OF LARGE ANIMAL SCIENCES
FACULTY OF HEALTH AND MEDICAL SCIENCES
UNIVERSITY OF COPENHAGEN
PHD THESIS 2014

ISBN 978-87-7611-772-6

HANNE KONGSTED

New Neonatal Porcine Diarrhoea Syndrome – A study on its aetiology, epidemiology and clinical manifestations

SL grafik